IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant :	Keith L. Black and Nagendra S. Ningaraj		
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Commissioner for Patents
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December 6, 2004

Declaration of Keith L. Black, M.D.

1. My name is Keith L. Black.

2. I received my Bachelor of Science (B.S.) in Biomedical Science with distinction from the University of Michigan at Ann Arbor in 1978.

3. I received my Medical Degree (M.D.) with distinction from the University of Michigan at Ann Arbor in 1981.

4. From 1981-1987 I completed my internship in General Surgery and then a residency in Neurological Surgery at the University of Michigan Medical Center.

5. From 1987-1991 I was an Assistant Professor in the Division of Neurosurgery at UCLA Medical Center in Los Angeles, California. I was promoted to Associate Professor in 1991, and full Professor in 1994.

6. In 1988, I became the Head of Neuro-Oncology at the UCLA Medical Center.

7. In 1992, I became the Ruth and Raymond Stotter Chair of the Department of Surgery at the UCLA Medical Center.

8. In 1995, I became a Professor of Neurology at the UCLA Medical Center.

9. In 1996, I became the Head of the UCLA Comprehensive Brain Tumor Program.

10. From 1997 until present, I have been affiliated with the Cedars-Sinai Medical Center in Los Angeles, California. At Cedars-Sinai, I serve as the Ruth and Lawrence Harvey Chair in Neuroscience, the Director of the Comprehensive Brain Tumor Program, the Director of the Division of Neurosurgery and the Director of the Cedars-Sinai Neurological Institute.

11. I am presently a Director of, and a scientific and medical advisor to Imagine Pharmaceuticals, Inc. a company engaged in blood brain barrier drug development.

12. I have been the recipient of numerous grants, including 8 NIH grants and a Robert Wood Johnson Foundation Faculty Award Grant.

13. I have received numerous awards and recognitions for my scientific and surgical expertise.

14. I have been an author on more than 250 scientific and medical publications.

15. I have been on the editorial board of the following scientific and medical journals: UCLA Cancer Trials; Critical Reviews in Neurosurgery; Perspectives in Neurological Surgery; the Journal of Neuro-Oncology; Neurological Research; the Journal of Radiosurgery; Gene Therapy and Molecular Biology; Neurosurgery Quarterly. I am also the editor of the internet publication Neuroscience Medicine and Technology (Net).

16. I am a named inventor on U.S. Patent Application No. 09/491,500 (the '500 application). I have read and understood this application.

17. I understand that the Examiner has rejected the pending claims in the '500 application on the grounds that they are not enabled by the specification. The '500 application teaches that modulators of K_{ATP} channels can enhance delivery of therapeutics from the blood specifically to abnormal or malignant cells.

14. Claim 1 of the '500 application is directed to a method of delivering a medicant to an abnormal brain region in a mammalian subject, comprising: administering to a mammalian subject having an abnormal brain region an agonist of an ATP-sensitive potassium channel, under conditions and in an amount sufficient to increase the permeability to the medicant of a capillary or arteriole delivering blood to cells of the abnormal brain region; and administering to the subject simultaneously or substantially simultaneously with the agonist the medicant, so that the medicant is delivered selectively to the cells of the abnormal brain region compared to normal brain regions.

The Method is Enabled to Treat Abnormal Brain Regions Generally

18. I understand that the Examiner suggests that it would be unpredictable whether the methods of the present invention could be used to treat any brain-based disease or disorder. Based on my years of research, I disagree for the following reasons.

19. I along with Dr. Nagendra Ningaraj, a researcher formerly affiliated with my laboratory at Cedars-Sinai Medical Center and a co-inventor of the present application, as well as Mamatha Rao, recently published a paper in the peer-reviewed journal Cancer Research that describes our laboratory observations relating to K_{ATP} expression in brain tumor cells and brain tumor capillary endothelium (Ningaraj NS, Rao MK, Black KL. *Adenosine 5'-triphosphate-sensitive potassium channel-mediated blood-brain tumor barrier permeability increase in a rat brain tumor model.* Cancer Res. (2003) 63(24):8899-911)

(see Exhibit A). This paper, along with a paper that we published last year in the Journal of Pharmacology and Experimental Therapeutics (featured on the journal cover), has been the subject of extensive interest on the part of scientists in the field (Ningaraj NS, Rao M, Hashizume K, Asotra K, Black KL. *Regulation of blood-brain tumor barrier permeability by calcium-activated potassium channels*. Journal of Pharmacology and Experimental Therapeutics (2002) 301: 838-851).

20. The invention is founded on our discovery that abnormal brain regions overexpress K_{ATP} channels, not only on abnormal brain cells but also on the epithelial cells which these abnormal brain cells contact. The fact that both types of cells have high levels of expression of these K_{ATP} channels is important to the targeting of the medicaments to the abnormal brain regions. Figure 1 of the Cancer Research paper (page 8902) shows a schematic representation of K_{ATP} channels in normal brain with neighboring endothelial cells as well as abnormal brain with neighboring epithelial cells. Blood tumor barrier cells and surrounding tumor cells are shown to overexpress K_{ATP} channels. Figure 1 also presents confocal microscopic immunolocalization of K_{ATP} channels (green) and von Willebrand factor (red) in normal brain capillaries and abnormal brain capillaries. Yellow indicates the co-localization of K_{ATP} channels in capillary endothelial cells. The results establish that K_{ATP} channels are hardly detectable in normal brain microvessel endothelial cells, while brain tumor capillaries overexpress K_{ATP}.

21. Our results further confirm that when an agonist of K_{ATP} is administered to brain tissue, the overexpressed K_{ATP} channels allow selective and enhanced delivery of compounds across brain tumor microvessels.

22. It was known prior to our discoveries that potassium channels can be overexpressed in certain non-brain tumor tissues, however, it was not until we performed our experiments that it was discovered that (i) primary brain tumor cells overexpress K_{ATP} channels; and (ii) brain microcapillary endothelial cells adjacent to the tumor also overexpress K_{ATP} channels.

23. Exhibit B (unpublished data) shows that K_{ATP} channels are overexpressed on metastatic brain tumors of diverse origin, as well as in microcapillary endothelial cells adjacent to those tumor cells. Specifically, Exhibit B shows that metastasized brain cancer cells originating from lung, breast and kidney cancers have high levels of K_{ATP} channels (left panels, green stain). Furthermore, these metastasized tumors also have high levels of expression of K_{ATP} channels on the brain microcapillary endothelium adjacent to the tumor (right panels, yellow cells, arrows).

24. Our results also confirm that when an agonist of K_{ATP} is administered to metastatic tumor tissue (breast and lung) in the brain, the overexpressed K_{ATP} channels allow selective and enhanced delivery of compounds.

25. Exhibit C (unpublished data) shows that potassium channel agonists enhance compound uptake in metastatic breast brain tumor. When rats are co-injected intravenously with [^{14}C]AIB and MS (a K_{ATP} agonist), the levels of [^{14}C] in tissue taken from the brain

tumor is approximately three times as great as if they are co-injected in saline and [^{14}C]AIB. The level of [^{14}C] in the brain tissue surrounding the tumor and in the contralateral brain does not differ significantly between the vehicle treated and MS treated rats.

26. Exhibit D (unpublished data) shows that potassium channel agonists enhance compound uptake in a metastatic lung brain tumor model. Intravenous co-injection of MS (a K_{ATP} agonist) increases the uptake of [^{14}C]AIB specifically to the tumor (versus the brain surrounding the tumor or contralateral brain] approximately three fold (black bars).

27. Not only have we shown that abnormal brain regions have high levels of K_{ATP} channels on both the malignant cells and on the endothelial cells, we have also shown that abnormal cells actually themselves enhance the expression of K_{ATP} channels on endothelial cells.

28. Figure 6 of the Cancer Research paper referenced above (page 8907) shows that specific binding of [^{3}H]-glibenclamide to endothelial cells is increased approximately 6-8 fold when the cells are co-cultured with glioma cells (compare panel A, lanes 3 to 5 or 8 to 10). [^{3}H]-glibenclamide binds specifically to K_{ATP} channels. These results suggest an increase in K_{ATP} channel density in the co-cultured cells. Consistent with these results, Figure 6 of the Cancer Research paper also establishes that the level of mRNA coding for a K_{ATP} channel subunit is enhanced in endothelial cells co-cultured with glioma cells. In the present of abnormal cells, mRNA for the K_{ATP} Kir 6.2 subunit is increased (Figure 6B, top panel, comparing lanes 1 to 3 or 5 to 4). This increase in level of mRNA translates into an increase in expression of the Kir 6.2 subunit protein (Figure 6C, comparing lanes b to c or d to f).

29. Figure 6 of the Cancer Research paper also shows that the K_{ATP} channels on the co-cultured endothelial cells are functional. The conductance of microcapillary brain endothelial cells is dramatically enhanced when the cells are co-cultured with tumor cells in the presence of the K_{ATP} agonist minoxidil sulfate (MS) (compare Figure 6D, plot 3 (peak at approximately -1500 RFU) to Figure 6E, plot 4 (peak at approximately -6000 RFU)). The conductance of the co-cultured cells is higher than that of tumor cells alone (see Figure 6E, plot 3 (peak at approximately -4000 RFU), which demonstrates the influence of tumor cells on the expression of the potassium channels on endothelial cells.

30. The results described in the Cancer Research paper indicate that localization of tumor cells near brain capillary endothelial cells increases the expression of functional K_{ATP} channels, which lead to selective increases in permeability.

31. Based on the data described above, and our extensive experience in this field, I conclude that we have discovered a method to selectively deliver a medicant to a wide range of abnormal brain tissue compared to normal brain tissue. This method gives hope to many patients suffering from presently incurable brain tumors and disorders.

The Method is Enabled for Medicants Generally

32. I understand that the Examiner suggested that the specification does not demonstrate delivery of an amount of any therapeutic agent sufficient to produce a therapeutic effect, and further, that the generalized delivery of macromolecules to the brain is precluded. Based on my years of research and clinical experience, I disagree for the following reasons.

33. A variety of compounds, including therapeutic macromolecules, have now been shown experimentally to be targeted to tumor cells by the potassium channel agonists described in the application.

34. Figure 3B of the Cancer Research paper referenced above shows that co-administration of minoxidil sulfate (MS) increases the uptake of [^{14}C]-labeled AIB, dextran and the chemotherapeutic agent carboplatin (CPN) (molecular weight= 371kD). Uptake of each of these compounds is enhanced only to the tumor center (black bars), as the level of ^{14}C is equivalent in MS treated and untreated surrounding brain (white bars) and contralateral brain (striped bars).

35. The Cancer Research paper also establishes that K_{ATP} agonists enhance delivery of gene therapy agents. Since the genetic components are encased in viruses, these agents are at least several million kilodalton (kD) in weight.

36. Specifically, Figure 5 of the Cancer Research paper shows that MS dramatically enhances the delivery of Her-2 monoclonal antibody to GBM tumor-bearing athymic nude rats (figure 5A, comparison of panel a to b and c) and Neu polyclonal antibody to rat glioma (RG2) tumor models (Figure 5A, comparison of panel d to e and f).

37. Figure 5B of the Cancer Research paper shows the ability of MS to increase delivery of adenoviral-green fluorescent protein (adv-GFP) to brain tumors compared adv-GFP infused alone (Figure 5B, compare panels g and j).

38. Our data also shows that the enhanced delivery of medicaments using KATP channel agonists results in therapeutically effective concentrations of medicament in the abnormal brain region. Figure 9 of the Cancer Research paper shows that tumor bearing rats treated with the combination of minoxidil sulfate (MS) and carboplatin (CPN) survived significantly longer than rats treated with vehicle alone. Figure 9A shows that the MS and CPN treated rats also survived longer than those treated with CPN alone. Furthermore, the MS and CPN treated rats showed smaller tumors on average than rats treated with vehicle alone or with CPN alone (see Figure 9B: MS + CPN (1.30 ± 1.2 mm^2); CPN alone (5.30 ± 1.9 mm^2); vehicle treated (9.37 ± 2.2 mm^2)).

39. In view of this data, and my experience in the field, I conclude that we have discovered a method to selectively deliver a wide variety of medicants to an abnormal brain tissue compared to normal brain tissue. This method will permit delivery of many therapeutic agents to the brain which have been used successfully to treat cancers in other parts of the body.

The K_{ATP} agonists can be administered in any manner that provides for delivery to the brain.

40. In support of the position that the claims are non-enabled, the Examiner has also suggested that the method or site of delivery can affect therapeutic outcome. The Examiner specifically notes that delivery of macromolecules of therapeutic values requires intracerebral injection of infusion by osmotic pumps.

41. Prior to the present discovery, the delivery of macromolecules to the brain *did* require the use of techniques like intracerebral injection, with attendant risks that limited the generalized use of therapeutic macromolecules. Our discovery, however, overcomes this limitation, to permit delivery of therapeutic macromolecules by less invasive techniques.

42. Our initial examples, described in the specification, utilized intra-carotid infusion. There is no reason, however, to believe that intracarotid delivery is required for the successful use of our method.

43. In fact, the K_{ATP} agonist can be administered in any manner that delivers the agonist systemically to the brain vasculature, including by intravenous, intraarterial and intracarotid delivery.

44. My group and I have performed several assays that show that intravenous infusion of the potassium channel agonist is also effective. The examples in Exhibit C and D show that intravenous infusion of potassium channel agonists is also effective in increasing the delivery of compounds to abnormal brain regions.

45. Exhibit C shows the delivery of [^{14}C]AIB to metastasized breast cancer tissue after intravenous injection of MS, which increased the delivery of AIB specifically to the tumor center by approximately three-fold over saline control.

46. Exhibit D shows the intravenous co-injection of [^{14}C]AIB with MS in a metastatic lung cancer model. Intravenous co-injection of MS increases the uptake specifically to the tumor center by approximately three fold (compare black bars).

Summary

46. In summary, we have shown that (i) K_{ATP} channels are overexpressed in primary brain and metastatic brain tumors tissue, including both the malignant cells and the capillaries embedded in the tumor; (ii) K_{ATP} agonists enhance the uptake of compounds in primary and metastatic brain tumors; (iii) normal brain does not have a high level of K_{ATP} channel expression and the channels that are expressed are not found on capillary endothelium; (iv) K_{ATP} agonists enhance the uptake of a wide variety of molecules in abnormal brain tissue, including therapeutic macromolecules; and (v) K_{ATP} channel agonists can be effectively delivered by a variety of methods to accomplish our method of treatment.

47. I declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true, and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, imprisonment, or both, under Section 1001, Title 18, United States Code, and that willful false statements may jeopardize the validity of this application and any patent issuing thereon.

Date: 12/6/2004

Keith L. Black, M.D.

Index of Exhibits

Exhibit A	Ningaraj NS, Rao MK, Black KL. *Adenosine 5'-triphosphate-sensitive potassium channel-mediated blood-brain tumor barrier permeability increase in a rat brain tumor model.* Cancer Res. (2003) 63(24):8899-911
Exhibit B	K_{ATP} channel expression on metastatic brain cancer cells and associated microcapillary endothelium
Exhibit C	Minoxidil sulfate (a K_{ATP} agonist) enhances compound uptake in metastatic breast brain tumor
Exhibit D	Minoxidil sulfate (a K_{ATP} agonist) enhances compound uptake in a metastatic lung brain tumor model

EXHIBIT A

[CANCER RESEARCH 63, 8899–8911, December 15, 2003]

Adenosine 5′-triphosphate-sensitive Potassium Channel-mediated Blood-Brain Tumor Barrier Permeability Increase in a Rat Brain Tumor Model

Nagendra S. Ningaraj, Mamatha K. Rao, and Keith L. Black

Maxine Dunitz Neurosurgical Institute and Burns and Allen Research Institute, Cedars-Sinai Medical Center, Los Angeles, California

ABSTRACT

Brain tumor microvessels/capillaries limit drug delivery to tumors by forming a blood-brain tumor barrier (BTB). The BTB overexpresses ATP-sensitive potassium (K_{ATP}) channels that are barely detectable in normal brain capillaries, and which were targeted for BTB permeability modulation. In a rat brain tumor model, we infused minoxidil sulfate (MS), a selective K_{ATP} channel activator, to obtain sustained, enhanced, and selective drug delivery, including various sized molecules, across the BTB to brain tumors. Glibenclamide, a selective K_{ATP} channel inhibitor, significantly attenuated the MS-induced BTB permeability increase. Immunocytochemistry and glibenclamide binding studies showed increased K_{ATP} channel density distribution on tumor cells and tumor capillary endothelium, which was confirmed by K_{ATP} channel potentiometric assay in tumor cells and brain endothelial cells cocultured with brain tumor cells. MS infusion in rats with brain tumors significantly increased transport vesicle density in tumor capillary endothelial and tumor cells. MS facilitated increased delivery of macromolecules, including Her-2 antibody, adenoviral-green fluorescent protein, and carboplatin, to brain tumors, with carboplatin significantly increasing survival in brain tumor-bearing rats. K_{ATP} channel-mediated BTB permeability increase was also demonstrated in a human, brain tumor xenograft model. We conclude that K_{ATP} channels are a potential target for biochemical modulation of BTB permeability to increase antineoplastic drug delivery selectively to brain tumors.

INTRODUCTION

Understanding the biochemical regulation of the blood-brain barrier (BBB) and blood-brain tumor barrier (BTB) is critical to developing methods to deliver therapeutic compounds to central nervous system targets. Despite evidence of the antineoplastic effect of drugs such as Trastuzumab, a humanized anti-Her-2 monoclonal antibody (MAb; developed by Genentech, Inc., San Francisco, CA), alone or with chemotherapeutic agents such as docetaxel (1, 2), their clinical use for neuro-oncology remains limited because of the high doses necessary to achieve *in vivo* therapeutically effective concentrations in brain tumors (2). Brain tumor capillaries constitute the BTB, which has different structural and functional characteristics to that of normal brain capillaries that form the BBB. Among many distinct differences (3), we showed that BTB capillaries are responsive to vasoactive agents (4–14). This knowledge allowed us to develop a biochemical approach to increase BTB permeability and to enhance delivery of hydrophilic therapeutic drugs or small- to large-sized molecules, including contrast-enhancing agents, antitumor compounds, therapeutic proteins, and viral vectors (4–14) *in vivo* to brain tumors selectively with little or no drug delivery to normal brain. This drug delivery strategy exploits the responsiveness of brain tumor capillaries to intravascular infusion of low doses of vasomodulators, such as bradykinin (BK), causing BTB permeability increase via a mechanism involving calcium-activated potassium (K_{Ca}) channels (4, 5), BK type 2 receptors (6), nitric oxide (7), and cyclic GMP (11). Overall, our research has identified the molecular targets that selectively modulate BTB permeability and contributed to a better understanding of the biochemical changes that occur during permeability modulations. More recently, we observed that another major potassium channel subtype, the ATP-sensitive potassium (K_{ATP}) channel, is involved in brain tumor microvessel permeability regulation and may serve as another target for anticancer drug delivery. We tested whether minoxidil sulfate (MS)-induced activation of K_{ATP} channels increases BTB permeability and enhances carboplatin (CPN) delivery to tumor tissue, thereby increasing survival in rats with implanted brain tumor. We demonstrated that CPN could be delivered selectively to tumor tissue without increasing delivery to normal brain cells.

K_{ATP} channels are heterodimers of sulfonylurea receptors and inwardly rectifying potassium channel subunits ($K_{ir}6.x$) with a (SUR-$K_{ir}6.x$)4 stoichiometry. K_{ATP} channels are widely distributed, including in the vasculature of the brain. They couple intracellular metabolic changes to the electrical activity of the plasma membrane regulating cerebral vascular tone and mediate the relaxation of cerebral vessels to diverse stimuli, including vasomodulators, in normal (15) and disease states (16). The role of K_{ATP} channels in regulating the permeability of normal and brain tumor capillaries, however, has not been elucidated. Brain tumors thrive in a hypoxic environment; this fact could explain the up-regulation of K_{ATP} channel expression detected in and around brain tumors but also shown in hypoxic and ischemic conditions (3, 16). Furthermore, endothelium-dependent regulation of cerebral blood vessel functions is impaired in brain tumors (17, 18), which might affect tumor capillary permeability in response to vasomodulators.

Brain tumors, particularly gliomas, frequently exhibit up-regulated epidermal growth factor receptor genes, which is associated with tumor aggressiveness, poor prognosis, and shortened patient survival (19, 20). One study found that 17–20% of primary brain tumors were Her-2 positive (20), and Her-2 has been linked to tumor progression. Mutant Her-2 MAbs directed against Her-2 or Her-2 inhibitors have been shown to block kinase activation of epidermal growth factor receptor through the formation of nonfunctional heterodimeric receptor complexes (21), which prevent tumor growth. Trastuzumab was developed to treat Her-2-positive breast cancers and may, possibly, also be effective in treating gliomas. Although Trastuzumab alone or in combination with a chemotherapeutic drug (22) has the potential to treat breast and lung cancers metastasized to brain, as well as gliomas, their efficient delivery across the BTB to tumor is highly challenging. Our strategy involves selective modulation of BTB permeability via activation of K_{ATP} channels in tumor capillaries to enhance delivery of Neu and Her-2 MAb in syngeneic and xenograft rat tumor models, respectively.

This study sought to elucidate the role of K_{ATP} channels in BBB and BTB permeability modulation and the role of transendothelial vesicular transport in normal and tumor capillaries in rats harboring intracranial rat gliomas (RG2). We investigated whether MS causes BTB permeability increase in a human, brain tumor xenograft model. We also studied whether brain tumor cells induce overexpression of K_{ATP} channels in brain endothelial cells cocultured with tumor cells.

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We further tested whether MS increases the transport of macromolecules, such as adenoviral vectors carrying green fluorescent protein (GFP) genes and Her-2 MAb, across the BTB in a human tumor xenograft model.

MATERIALS AND METHODS

MS, glibenclamide (Sigma Chemical Co., St. Louis, MO), 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H benzimidazol-2-one (NS-1619), iberiotoxin (RBI Chemical, Natik, MA), membrane potential assay kit (Molecular Devices, Sunnyvale, CA), and radiotracers (NEN Co., Boston, MA) such as [^{14}C] α-aminoisobutyric acid ([^{14}C]-AIB; 57.6 mCi/mmol; M_r 103,000), [^{14}C]-CPN (25 mCi/mmol; M_r 371,000), [^{14}C] dextran (2 mCi/mmol; M_r 70,000), [^{3}H]-glibenclamide (50 mCi/mmol; M_r 494,000), von Willebrand factor (vWF; DAKO, CA), Neu polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), glial fibrillary acidic protein (GFAP; Chemicon, Temecula, CA), Her-2 MAb (clone CB 11; Zymed, San Francisco, CA), and fluorescent-labeled secondary antibodies (Chemicon; and Molecular Probes, Eugene, OR) were used in the present study.

In Vivo BBB/BTB Permeability. All animal experiments were conducted in accordance with policies set by the Institutional Animal Care and Use Committee and NIH guidelines. A rat syngeneic tumor model was prepared using female Wistar rats, and a human tumor xenograft model in athymic nude rats weighing 180–200 g was prepared for the BBB/BTB permeability studies. Although brain tumor blood vessel development is rapid in rats compared with humans, this variable should not affect our findings or conclusions, because our study analyzes the response of blood vessels, regardless of how rapidly they developed, to vasomodulators. The optimum number of tumor cells and incubation period for *in vivo* tumor growth was determined in separate experiments. RG2 cells (1×10^5) in 5 μl of medium with 1.2% methylcellulose were injected into the basal ganglia of Wistar rats, whereas nude rats received injections of glioblastoma multiforme (GBM) primary cells (5×10^5). The coordinates were 5 mm lateral to the bregma and 4.5 mm deep to the basal ganglia. Seven days (for RG2 tumor) and 3–4 weeks (for GBM) after tumor implantation, the rats were prepared for permeability study, as described previously (4, 5). In regional permeability studies, 5 min after the start of the intracarotid (i.c.) infusion, 100 μCi/kg [^{14}C] AIB, [^{14}C] dextran, or [^{14}C]-CPN in 1 ml of phosphate buffer saline (PBS) were injected as an i.v. bolus within a 15-s period. To determine whether the infusion of vasomodulators would affect cerebral blood flow (CBF) at the tumor area, we performed cerebral laser-Doppler flowmetry (LDF) using a laser-Doppler (DRT4; Moore Instruments Ltd., Devon, United Kingdom) equipped with a DP3 optical (1 mm diameter) probe during a 15-min i.c. infusion of vasomodulators in some rats (n = 3/group), as described previously (4, 5). Rats with abnormal CBF, blood gases, or blood pressure were excluded from the study.

K_i Measurement. The unilateral transfer constant K_i (μl/g/min), which is an initial rate for blood-to-brain transfer of a radiotracer, was calculated as described by Ohno *et al.* (23). The K_i was determined for radiotracers [^{14}C]-AIB, [^{14}C]-CPN, and [^{14}C] dextran in the tumor core, tumor-adjacent brain tissue, and contralateral brain tissue using the quantitative autoradiographic (QAR) method as described previously (4, 5). An optimum dose (10 μg/kg/min, i.c.) of BK, which was established in previous studies (4, 5, 9–11), and optimal dose (30 μg/kg/min for 15 min, i.c.) of MS were used for K_i measurements in rats (n = 6/group). In a separate QAR study, various doses of glibenclamide (0–10 μg/kg/min) were used to establish an optimal dose, which maximally attenuated the MS-induced BTB permeability increase in RG2 tumor-bearing rats. Additional experiments were performed in rats with RG2 tumor by coinfusing MS with glibenclamide (5 μg/kg/min) to investigate whether inhibition of K_{ATP} channels by glibenclamide attenuates MS-induced permeability increase. In addition, the effect of MS [with optimum dose and time established in rats (n = 4/dose) harboring intracranial RG2 tumor] on BTB permeability in nude rats harboring GBM was investigated.

Dose-Response Study. To establish the optimal and safe dose range that would result in selective increases in BTB permeability without appreciably altering systemic blood pressure, various doses (0–60 μg/kg/min) of MS were administered. In a separate study, Evans blue dye was administered i.v. in rats (n = 4/group) with RG2 tumors to achieve a semiqualitative measure of BTB permeability increase.

Time Course. To determine whether vasomodulator-induced BTB permeability increase in RG2 tumor-bearing rats (n = 4/time period) was transient or could be sustained over a long period, a separate QAR study was performed. BK (10 μg/kg/min), MS (30 μg/kg/min), or a K_{Ca} channel activator (NS-1619) was infused (i.c.) separately for 15-, 30-, and 60-min periods, and K_i was determined as described above.

Synergistic Effect of K_{Ca} and K_{ATP} cChannel Agonists on BTB Permeability. To investigate whether MS-induced BTB permeability increase was independent of K_{Ca} channels, MS was coinfused with the K_{Ca} channel inhibitor iberiotoxin in RG2 tumor-bearing rats (n = 4/group). Furthermore, to investigate whether K_{Ca} and K_{ATP} channel agonists administered simultaneously would exert a synergistic effect on BTB permeability, NS-1619 and MS (30 μg/kg/min each) were coinfused (i.c.) for 15 min, and K_i was measured by QAR.

Isolation of Brain Endothelial Cells. To investigate the expression and activity of K_{ATP} channels in rat brain endothelial cells (RBECs), we isolated endothelial cells from the brains of neonatal rats using the method described previously (4). The homogeneity (>90–95%) of endothelial cells was verified by immunostaining with an endothelial cell marker, factor VIII/vWF. For potentiometric assays, RBECs were seeded alone and in coculture with RG2 tumor cells in a gelatin-coated, 96-well plate to obtain a monolayer.

Coculture Experiments. To investigate whether brain tumor cells can induce overexpression of K_{ATP} channels in RBECs and human brain microvessel endothelial cells (HBMVECs), we cocultured RBECs with RG2 cells and HBMVECs with GBM cells. We standardized suitable conditions for the growth of the coculture on glass coverslips in 6-well tissue culture plates. Initially, ~1×10^4 cells of RBECs and HBMVECs were cocoated with 1×10^4 cells of RG2 and GBM, respectively, and allowed to achieve 70% confluency. Reverse transcription (RT)-PCR and Western blot analyses in RBECs and HBMVECs alone or in coculture with tumor cells were performed to study whether tumor cells induce overexpression of K_{ATP} channels at mRNA and protein levels in endothelial cells. In addition, immunocytochemical analysis of cocultures was performed with vWF and K_{ATP} channel antibodies to support RT-PCR and Western blot data.

Western Blot Analysis. To investigate the differential expression of K_{ATP} channels by the Western blot method, protein homogenates of normal brain and tumor tissues, tumor cells alone, or in cocultures were prepared by rapid homogenization in 10 volumes of lysis buffer [1% SDS, 1.0 mM sodium vanadate, 10 mM Tris (pH 7.4)]. The extracts of normal brain and tumor tissues obtained from nude rats, which harbored intracerebral glioma for 3–4 weeks after tumor cell implantation, were used. Immunoblot analysis was performed on RG2 and GBM cells. Control protein lysates for the protein/receptor of interest were purchased from various vendors. Samples were fractionated on a 6–12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and probed with respective affinity-purified antibodies (optimum dilution was determined) for 1 h. After incubating with the primary antibody, membranes were incubated with peroxidase-conjugated rabbit antimouse/rabbit IgG for 1 h. The signals were detected with an enhanced chemiluminescence kit (Amersham Biosciences Corp., Piscataway, NJ). Rabbit polyclonal primary anti-K_v6.2 antibody, as described by Lorenz *et al.* (24), was raised against the peptide sequences (EDP AEP RYR ARQ RRA RFV SKK). Anti-β-actin MAb was obtained from Santa Cruz Biotechnology.

Immunocytochemistry (ICC). We colocalized K_{ATP} channels in rat and human brain and tumor sections with an anti-K_{ATP} channel antibody raised against a K_v6.2 subunit that was shown to be present in brain along with sulfonylurea receptor 2A/B (16, 25). ICC and confocal-laser-scanning microscope analysis were used to detect colocalization and differential expression of K_{ATP} channels with K_v6.2 antibody in normal and brain tumor microcapillary and in human and rat tumor tissues. In ICC studies of K_{ATP} channels, HBMVECs and COS cells were positive and negative controls, respectively. All ICC experiments included either a control sample preincubated with a respective blocking peptide, whenever available, or control samples that did not contain primary antibodies. ICC analysis of K_{ATP} channels in athymic nude rats with intracerebral GBMs was performed as described recently (4, 5). To determine whether K_{ATP} channels are present on capillary endothelial and tumor cells, brain tumor sections were incubated with polyclonal K_v6.2 and monoclonal anti-vWF primary antibodies. The colocalization of K_{ATP} channels and vWF antibodies was accomplished with antirabbit IgG conjugated with a fluorescent

(FITC) and antimouse IgG conjugated with rhodamine (tetramethylrhodamine isothiocyanate). To demonstrate the expression of K_{ATP} channels in endothelial, RG2, and GBM tumor cells, monolayers of RG2 and GBM cells were fixed with 4% paraformaldehyde, processed similarly, and immunostained with anti-K_v6.2 primary antibody and antirabbit IgG conjugated with FITC. To demonstrate that tumor cells *in vitro* and *in vivo* were of glial origin, RG2 and GBM cells were immunostained with antirabbit polyclonal GFAP and antirabbit IgG conjugated with tetramethylrhodamine isothiocyanate. Immunostaining of rat brain sections after i.c. delivery of Neu (human Her-2 equivalent) in RG2-bearing rats was performed with antirabbit Neu and secondary antirabbit IgG conjugated with tetramethylrhodamine isothiocyanate. In contrast, immunostaining of rat brain sections after i.c. delivery of Her-2 MAb in GBM tumor-bearing rats was detected using a Zenon Immunostaining kit (Molecular Probes).

RT-PCR Analysis. RNA was isolated from endothelial (HBMVECs, RBECs), and tumor (RG2, GBM) cells and tissue samples and purified using TRIzol (Invitrogen Life Technologies, Inc., Carlsbad, CA), following the manufacturer's instructions. First-strand synthesis of cDNA using random hexamers was performed in 2-μg aliquot of DNase I-treated total RNA using Superscript II. Subsequently, 4 μl of cDNA were amplified in a standard 50-μl PCR reaction (at appropriate conditions) containing 0.12 μmol/liter of each primer. An aliquot (20 μl) of the RT-PCR product was analyzed on a 1% agarose gel and visualized using ethidium bromide. The locations of the primers indicated are based on the subunit sequence information obtained from GenBank: K_v6.2 subunit of K_{ATP} channel (human; accession no. D50582). The following primers were used for amplification of K_v6.2 subunit of K_{ATP} channel (5′-GTCACCGGAGCCATGCTGTCCCGC-3′ and 5′GGGGGCCCGAGAGACCATGGCTCA-3′), and β-actin (5′-AATCTGGCACCACACCTTCTAC-3′ and 5′ CTTCTCCTTAATGTCACGCACG-3′).

Confocal Microscopy. Immunoreactive visualization of K_{ATP} channels, Her-2, and vWF was performed as described previously (4, 5). In brief, confocal images were captured using a Leica (Heidelberg, Germany) True Confocal Scanner Spectrophotometer laser scanning confocal microscope (inverted) equipped with argon (488 nm) and krypton (568 nm) lasers. Fluorescent signals for K_{ATP} and Neu/Her-2 expressed on cultured rat and human endothelial cells, brain tumor cells, and brain tumor-bearing rat brain sections were visualized using the 488-nm argon laser line, and fluorescent signals on vWF were visualized using the 568-nm krypton laser line. Fluorescence signals for flour-488 or -568 were displayed individually as green and red pseudocolor projections or merged as overlay projections to visualize possible colocalization of two different antigens within specific subcellular structures.

[^{3}H]-Glibenclamide Binding Study. The densities of K_{ATP} channels in rat brain endothelial and tumor cells and in human normal brain and tumor tissue were determined by [^{3}H]-Glibenclamide binding study. To quantify K_{ATP} channels *in vitro*, RBECs alone or cocultured with RG2 cells, rat brain, RG2 tumor tissue, HBMVECs alone or cocultured with GBM, and normal human brain and GBM tissues were incubated with glibenclamide (5 μM) and [^{3}H]-glibenclamide (5 nM) for 1 h in sodium phosphate buffer (10 mM, pH 7.4). The assay was modified from Challinor-Rogers *et al.* (25). Samples were washed three times with sodium phosphate buffer, and cells/tissue were lysed with ice-cold sodium phosphate buffer, mixed with liquid scintillation fluid, and counted on a liquid scintillation counter. The actual binding of [^{3}H]-glibenclamide was calculated by measuring the difference between nonspecific and specific bindings in three separate experiments conducted in duplicate.

Membrane Potential Assay. Functional activities of putative K_{ATP} channels in HBMVECs and GBM cells were measured by detecting changes in voltage across the cell membrane, as described previously (4). We used the membrane potential assay kit (Molecular Devices), which provided a fast, simple, and consistent mix-and-read procedure. In brief, HBMVECs and GBM cells obtained from cell passages 2–5 were suspended in 5 ml of growth medium. In two separate experiments, the cell density was adjusted to 1×10^5 cells/well and coated in sterile, clear-bottom, black 96-well plates (Corning Inc., Acton, MA), precoated with poly L-lysine, and allowed to achieve a monolayer within 24 h. The monolayer cells were incubated with the membrane potential assay kit reagents for 30 min and read directly by FLEXstation. The anionic potentiometric dye that traverses between cells and the extracellular solution in a membrane potential-dependent manner serves as an indicator of vasomodulator-induced voltage changes across the cell membrane. To determine the optimum dose that elicits detectable voltage changes across HBMVEC and GBM cell membranes, dose-response studies were performed with 0–50 μM K_{ATP} channel agonist MS and K_{ATP} channel antagonist glibenclamide (100 nM), using the Spectrofluorometer set to the following parameters: excitation (530 nm), emission (565 nm); and emission cutoff (550 nm) wavelengths. Additionally, the effects of the optimum dose (10 μM) of MS and glibenclamide (100 nM) on K_{ATP} channel activity in HBMVECs and GBM cells were determined.

GFP-Adenoviral (GFP-Adv) and erbB-2 Antibody Delivery. *GFP*-Adv constructs were prepared as described by Smith *et al.* (26), with slight modifications. The *GFP* gene was first cloned into a shuttle vector, pAdTrack-CMV, and the resultant plasmid was linearized by digesting it with restriction endonuclease Pme I and subsequently cotransformed into *Escherichia coli* cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinant plasmids were transfected into 293 cells. The presence of recombinant adenoviruses was verified by RT-PCR. *GFP*-Adv (1×10^9 pfu/ml) was infused i.c. with or without MS in rats with implanted GBM. Neu rabbit polyclonal and Her-2 MAb, which are specific for rat and human erbB-2 receptors, respectively, were used in this study. Both antibodies were dialyzed using M_r 100,000 cutoff dialysis tubing (0.5-ml capacity, 10-μm pore size; Spectrum Laboratories) to remove BSA and other additives. MS (30 μg/kg/min) was infused (i.c.) for 15 min, followed by i.c. infusion of Neu or Her-2 MAb (1 mg/ml/kg) or GFP-Adv (1×10^9 pfu/ml) for 45 min. After 2 h, rats ($n = 2$) were perfuse fixed with 4% paraformaldehyde transcardially, and the brain removed. Her-2 MAbs bound to Her-2 receptors *in vivo* was detected by incubating rat brain cryosections as per the manufacturer's (Molecular Probes) procedure with Alexa Flour-647-conjugated secondary antibody (Zenon labeling kit) that specifically binds to the IgG1 fragment of Her-2 MAb. Neu antibody binding to erbB-2 receptors in RG2 brain tumor sections was detected using antirabbit IgG conjugated with tetramethylrhodamine isothiocyanate. To investigate whether there was Adv-*GFP* delivery and expression of GFP in tumor cells, another group of rats ($n = 2$) were transcardially perfuse fixed with 4% paraformaldehyde after 96 h, brains were removed, and cryosections were imaged for the presence of GFP by confocal microscopy. Furthermore, to demonstrate the expression of GFP on glial tumor cells, GFP was colocalized with GFAP.

Transmission Electron Microscopy. Seven days after RG2 tumor cell implantation, rats ($n = 3$/group) were infused i.c. with PBS, BK (10 μg/kg/min in 0.5% DMSO), NS-1619 (30 μg/kg/min in 0.5% ethanol), or MS (30 μg/kg/min in 0.5% DMSO) for 15 min. Rats were infused with 10 ml of cold PBS and perfuse fixed with 250 ml of 1% glutaraldehyde in PBS (pH 7.4) through the heart. Tumor-bearing brains were removed, and 1-mm^3 tissue pieces encompassing tumor mass, brain surrounding the tumor, and normal brain were cut, and samples were processed for transmission electron microscopy analysis (JEOL electron microscope operating at 80 kV), as described previously (4).

Quantitative Analysis. At least 10 profiles of capillaries from each group sectioned transversely and photographed at low magnification (×7200) were evaluated for their general features, as described previously (4). Briefly, micrographs were placed on a digitizing screen, and structural features were measured using Scan Pro 4, a computer-assisted, image analysis system (Jandel Scientific, Corte Madera, CA). Abluminal and luminal circumferences, areas of endothelial cytoplasm excluding nuclei and vacuoles, and mean thickness of endothelial cytoplasm were measured and compared with those in the control group. The mean thickness of endothelium was calculated by subtraction of the luminal radius from the abluminal radius, which was obtained from the areas encircled by the luminal and abluminal circumferences, respectively. The proportion of total vesicular area to the cytoplasmic area was expressed as a percentage to derive another parameter to characterize vesicular transport.

Survival Study. Wistar rats with implanted intracranial tumors were used to study the effect of MS on increased CPN delivery and survival. RG2 cells (1×10^5) were implanted intracranially to form a tumor (2 mm in size) in rats within 1 week. After 1 week, rats were given saline, CPN (5 mg/kg), or CPN after 15 min of MS (30 μg/kg/min for 15 min) infusion through an exteriorized catheter once a day for 3 consecutive days. The rats were monitored carefully for mortality and clinical signs attributable to brain tumor growth for 90 days or until death, whichever came first. Brains of dead or moribund rats or those rats that survived beyond 90 days were removed, frozen, and cryosectioned for histological evaluation, to compare tumor volumes between treated and un-

treated groups. Kaplan-Meier analysis was performed to determine the statistical significance of this study.

Statistics. Results are expressed as mean ± SD, where applicable. For all *in vivo* permeability studies, we used $n \geq 4$ rats/group, unless stated otherwise. Unpaired two-tailed Student's *t* tests were used to compare the control and treated groups. The statistical analyses of K_i, vesicle density, vesicular area, cleft index, and cleft area index comparison among different groups, with or without drug treatment, were performed using ANOVA, followed by either unpaired parametric analysis of Student's *t* test or by nonparametric analysis of the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant. Statistical analysis of the vesicular density and proportion of cumulative vesicular area compared the effect of i.c. infusion of MS with that of PBS infusions.

RESULTS

BBB Capillaries Differ from BTB Capillaries. Fig. 1 shows the differences in the response of the BBB and BTB to K_{ATP} channel agonists. BTB capillaries and surrounding tumor cells overexpress K_{ATP} channels and, therefore, may readily respond to activation by K_{ATP} channel agonists. In contrast, K_{ATP} channels are hardly detected in normal brain microvessel endothelial cells, which, therefore, may not respond to K_{ATP} channel agonists.

K_{ATP} Channels Mediate MS-Induced BTB Permeability Increase. BTB permeability, K_i (μl/g/min), was measured by QAR of cryosections obtained from RG2 or GBM tumor-bearing rat brains after the injection of a [^{14}C]-labeled tracer, 5 min after i.c. MS infusion. To determine whether K_{ATP} channels mediate MS-induced BTB permeability increase, rats with implanted intracerebral RG2 tumors received i.c. MS infusion alone or with glibenclamide. K_i was determined for radiotracer [^{14}C]-AIB in the tumor core, tumor-adjacent brain tissue, and contralateral brain tissue. Comparison of pseudocolor-enhanced autoradiographs of rat brain sections showed enhanced delivery of [^{14}C]-AIB on i.c. MS infusion (Fig. 2*A*). Glibenclamide coinfusion blocked MS-induced [^{14}C]-AIB uptake (Fig. 2*A*). After i.c. infusion of MS (30 μg/kg/min) for 15 min, K_i significantly increased in the tumor center (32 ± 5 μl/g/min; $P < 0.001$) compared with PBS controls (10.5 ± 1.5 μl/g/min). MS-induced increase in BTB permeability was significantly inhibited (12.2 ± 3.0 μl/g/min; $P < 0.01$) by coinfusion of glibenclamide (5 μg/kg/min for 15 min; Fig. 2*B*). MS-induced K_i increase was not blocked by iberio-

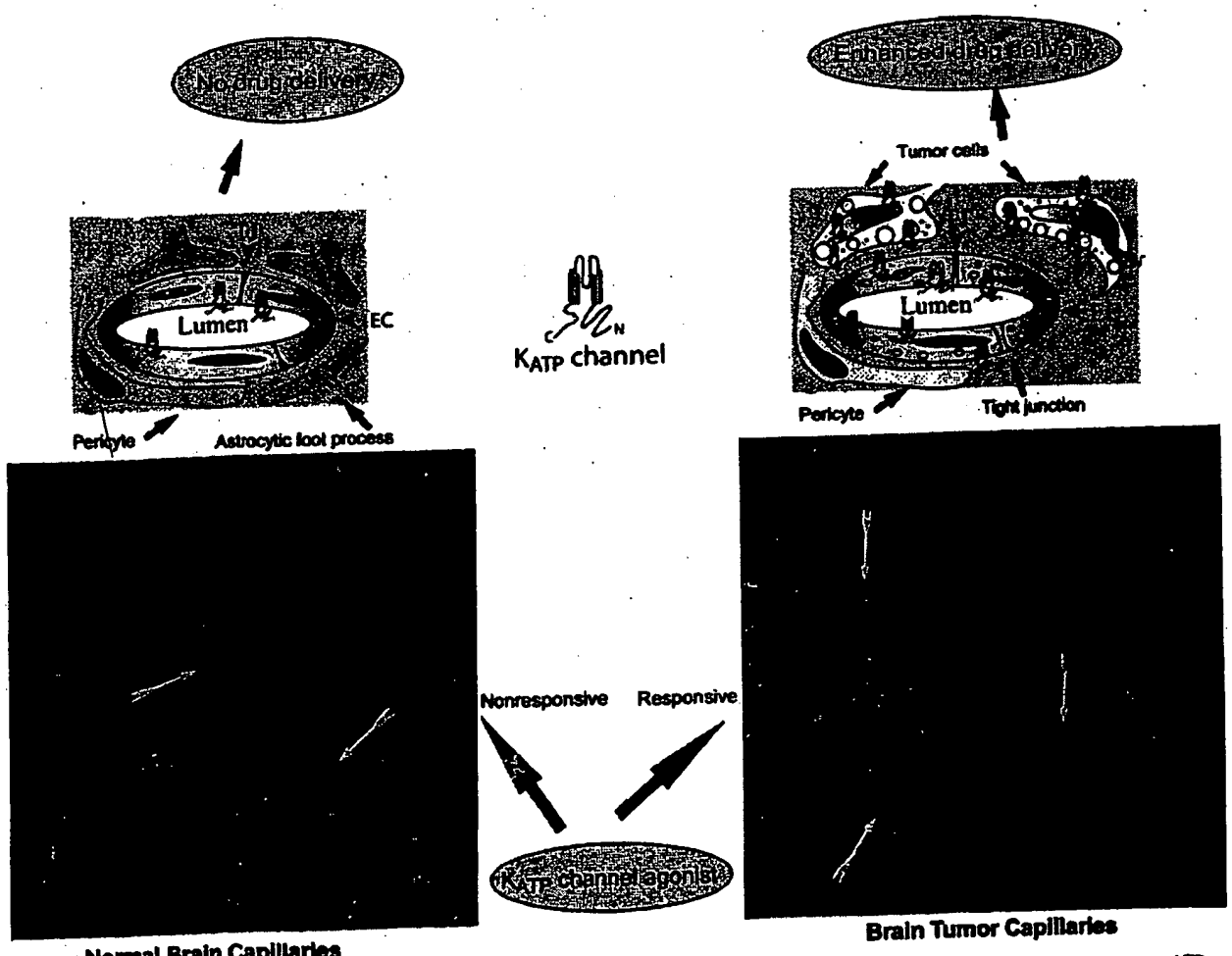

Fig. 1. Schematic representation. The differences between the blood-brain barrier and blood-tumor barrier with regard to their morphology and biochemical response to ATP-sensitive potassium (K_{ATP}) channel agonists are shown. blood-tumor barrier capillaries and surrounding tumor cells overexpress K_{ATP} channels and, therefore, may readily respond to activation by K_{ATP} channel agonists, resulting in enhanced formation of transport vesicles in tumor capillary endothelial and tumor cells. Confocal microscopic immunolocalization of K_{ATP} channels (green) and von Willebrand factor (red) in human normal brain, tumor capillary endothelial cells, and tumor cells was performed using $K_{ir}6.2$ and von Willebrand factor antibodies. Yellow indicates the colocalization of K_{ATP} channels in capillary endothelial cells. Brain tumor capillaries overexpress K_{ATP} channels and were colocalized with von Willebrand factor on endothelial cells. In contrast, K_{ATP} channels are hardly detected in normal brain microvessel endothelial cells and may be nonresponsive to K_{ATP} channel agonists. Our strategy involves biochemical modulation of K_{ATP} channels for selective and enhanced drug delivery to tumors with little or no drug delivery to normal brain tissue. *EC*, endothelial cell; *TJ*, tight junction.

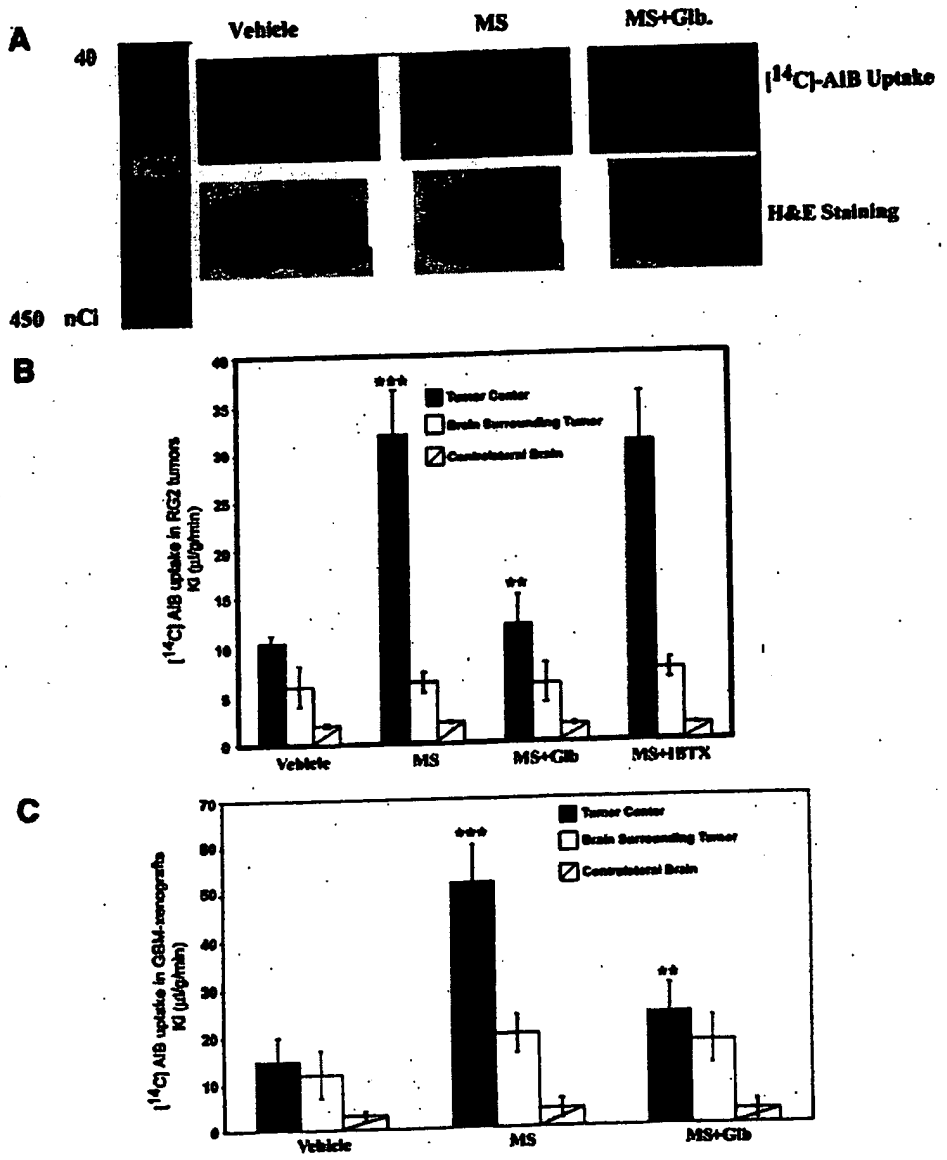

Fig. 2. Quantitative increases in blood-tumor barrier permeability. *A*, color-enhanced autoradiographs of coronal brain sections showing little [^{14}C] α-aminoisobutyric acid (*[^{14}C]-AIB*) delivery in a rat with an intracranial rat glioma (*RG2*) tumor after i.c. vehicle (PBS + 0.5% DMSO) infusion but significantly enhanced delivery in an MS-treated rat. The MS-induced increase in [^{14}C]-AIB delivery was significantly diminished when glibenclamide (*Glb*) was coinfused. For comparison, the scale on the *left* shows pseudocolor intensities of tissue-calibrated [^{14}C] standards from 40–450 nCi/g-specific activities. *B*, the mean K_i for [^{14}C]-AIB significantly increased after i.c. infusion of minoxidil sulfate (*MS*; 30 μg/kg/min for 15 min) compared with a vehicle-treated group. The K_i increase was significantly attenuated by coadministration of Glb ($n = 4$). Coinfusion of a K_{Ca} channel inhibitor, iberiotoxin (*IBTX*), with MS failed to block the MS-induced K_i increases. *C*, in addition, MS (30 μg/kg/min for 15 min) enhanced blood-tumor barrier permeability in a glioblastoma multiforme (*GBM*) xenograft model compared with a PBS-treated group and was significantly attenuated by coadministration of Glb. Data are presented as mean ± SD ($n = 4$). **, $P < 0.01$ *versus* MS-treated group; ***, $P < 0.001$ *versus* vehicle group.

toxin (a specific K_{Ca} channel inhibitor), suggesting that the action of MS is independent of K_{Ca} channels (Fig. 2*B*). In a separate study, we found that the increase in BTB permeability obtained with a fixed dose of MS (30 μg/kg/min) was blocked by coinfusion with glibenclamide (0–25 μg/kg/min) in a dose-dependent manner (data not shown). Additionally, in a GBM xenograft nude rat model, we showed that MS (30 μg/kg/min) also significantly increased BTB permeability (52 ± 8 μl/g/min; $P < 0.001$), which was attenuated (24 ± 6 μl/g/min; $P < 0.01$) when glibenclamide (5 μg/kg/min for 15 min) was coinfused with MS (Fig. 2*C*). In contrast to the tumor center, MS with or without glibenclamide did not significantly affect BBB permeability in normal brain surrounding tumor (2-mm area outside tumor margin) or in normal contralateral brain (Fig. 2*C*). Similarly, when infused alone or before MS infusion, glibenclamide did not affect BBB or BTB permeability (data not shown).

Time Course. The present QAR study in rats with implanted RG2 tumor showed that i.c. infusion of MS (30 μg/kg/min) significantly ($P < 0.001$) enhanced sustained delivery of [^{14}C]-AIB to the tumor for 15-, 30-, and 60-min infusions (Fig. 3*A*). We found that the ability of MS to sustain BTB permeability increase up to 60 min was consistent with our reported data on NS-1619 in a similar model (4). In contrast, a 30- or 60-min infusion of BK failed to sustain the initial increase of K_i ($P < 0.001$) attained at 15 min (Fig. 3*A*). For comparative purpose, we infused a similar molar concentration of BK and NS-1619.

K_{ATP} Channel Activation Elicits Increased Delivery of Molecules of Various Sizes. Intracarotid infusion of MS (30 μg/kg/min) increased BTB permeability in implanted intracerebral RG2 tumor to various-sized radiotracers that normally fail to cross the BTB, including hydrophilic compounds such as [^{14}C]-labeled- AIB, dextran, and a chemotherapeutic agent, CPN. Coinfusion with MS significantly enhanced delivery of [^{14}C]-labeled AIB, dextran, and CPN (Fig. 3*B*). In contrast, [^{14}C]-CPN delivery was negligible in vehicle-treated rats. These studies further suggest that K_{ATP} channel-mediated increases in BTB permeability allow delivery of drugs of various molecular sizes, including AIB (M_r103,000), CPN (M_r 361,000), and dextran (M_r 70,000), suggesting that the effect is independent of molecular size (Fig. 3*B*).

Synergistic Effect of K_{Ca} and K_{ATP} Channel Agonists on BTB Permeability. To investigate whether K_{ATP} and K_{Ca} channel agonists exert a synergistic effect on BTB permeability increase, MS and NS-1619 were coinfused i.c. for 15 min. This combination signifi-

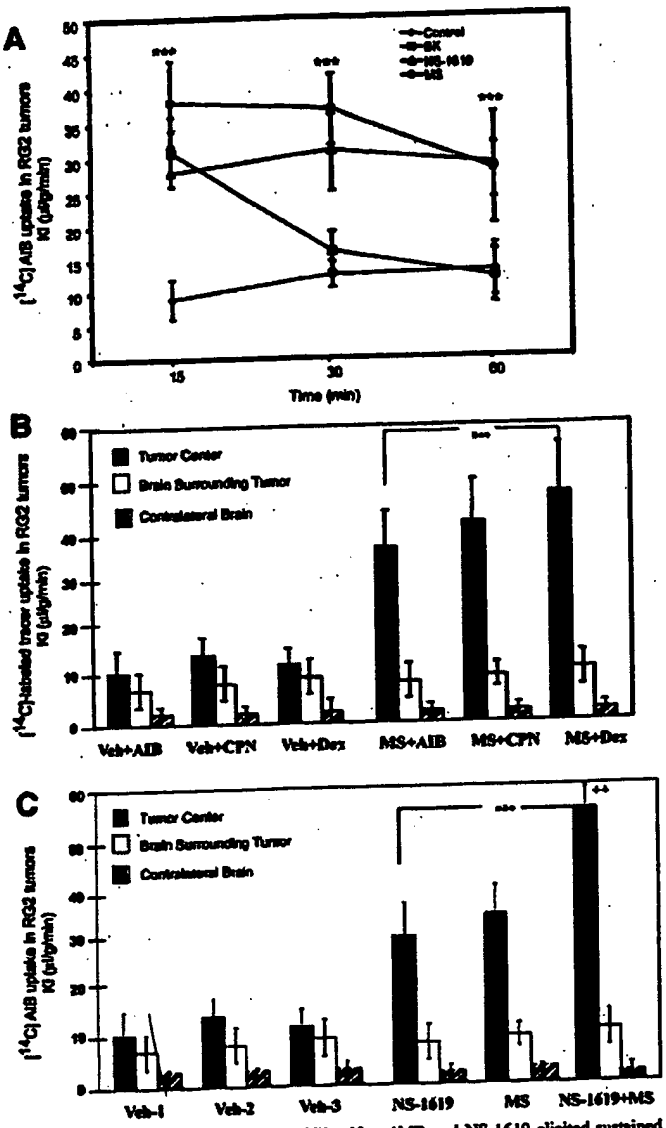

Fig. 3. Time course study. *A*, minoxidil sulfate (*MS*) and NS-1619 elicited sustained increases in mean K_i values for up to 30 and 60 min, respectively. In contrast, bradykinin (*BK*)-induced K_i increase was transient and lasted for 15–20 min. Prolonged BK infusion (up to 60 min) failed to sustain the K_i increase. Data in *A–C* are presented as mean ± SD. ***, $P < 0.001$ *versus* vehicle group ($n = 4$). B, K_i values within rat glioma (*RG2*) tumors comparing blood-tumor barrier permeability to radiotracers of different sizes including aminoisobutyric (*AIB*), carboplatin (*CPN*), and dextran (*Dex*) with and without MS infusion. The K_i values in the MS-treated groups were significantly (***, $P < 0.001$) higher than in the vehicle (*Veh*)-treated group. *C*, we observed a synergistic effect of MS with NS-1619 (i.c.) in RG2 tumor-bearing rats. The K_i value for [^{14}C]-AIB delivery after combination treatment was significantly (++, $P < 0.01$) higher compared with groups treated with MS or NS-1619 alone. *Veh-1*, PBS + 0.5% DMSO; *Veh-2*, PBS + 0.5% ethanol; *Veh-3*, PBS + 0.5% DMSO and ethanol.

cantly increased BTB permeability compared with the BTB permeability increase elicited when the drugs were infused alone (Fig. 3*C*). This result suggests that K_{ATP} channel-mediated BTB permeability increase occurs by a different pathway than the K_{Ca} channel-mediated effect.

ErbB-2 Antibody and GFP-Adv Delivery across the BTB. ErbB-2 expression was demonstrated in RG2 and GBM *in vitro* and *in vivo* (Fig. 4). Low Her-2-expressing MCF-7 (human breast tumor cells) were used as a negative control. Furthermore, the glial origin of RG2 and GBM was demonstrated by GFAP expression *in vitro* and *in vivo* (Fig. 4*B*). After studying the ability of MS to increase BTB permeability to allow the delivery of various-sized molecules, we investigated whether MS increases Neu, Her-2 MAb, and GFP-Adv delivery to rat brain tumor *in vivo*. In this study, we used RG2 (Neu positive) tumor-bearing Wistar rats and GBM (Her-2 positive) tumor-bearing athymic nude rats. MS infusion enhanced delivery of Her-2 MAb selectively to GBM and of Neu to RG2 (Fig. 5*A*) tumor tissues without any delivery to contralateral brain tissues (Fig. 5*A*). In contrast, very little Her-2 MAb or Neu was delivered to tumor tissue in vehicle-treated rats (Fig. 5, *Aa* and *Ad*). MS also enhanced adv-GFP delivery to brain tumors. Abundant GFP expression was seen in brain tumor cells in rats coinfused with MS and adv-GFP (Fig. 5*Bg*) but not in the tumor periphery (*TP*). In addition, GFP expression was observed predominantly on the tumor cells because GFP colocalized with GFAP (Fig. 5*Bi*). In contrast, adv-GFP infused alone failed to cross the BTB and, therefore, negligable GFP expression was detected on tumor cells. Furthermore, GFP expression was observed in the cerebral blood vessels but not in the tumor cells because the GFP did not colocalize with GFAP (Fig. 5*Bl*).

K_{ATP} Channel-mediated BTB Permeability Modulation and CBF. Previously, we showed that i.c. infusion of low doses of K_{Ca} channel activators such as BK and NS-1619 did not alter CBF in tumor and normal brain (4). In the present study, using a laser-operated Doppler, we showed that i.c. administration of 30 μg/kg/min MS did not significantly affect CBF (Table 1), although BTB permeability increased in the tumor area. Mean arterial blood pressure in the drug-treated groups was not significantly different from the vehicle-treated group (Table 1).

Tumor Cells Increase K_{ATP} Channel Expression in Endothelial Cells. The specific binding of [^{3}H]-glibenclamide in the membranes of tumor cells and tissues was significantly higher than in membranes prepared from normal endothelial cells and brain tissue (Fig. 6*A*). When cocultured with tumor cells, endothelial cells showed increased [^{3}H]-glibenclamide binding compared with endothelial cells and tumor cells alone (Fig. 6*A*), suggesting an increase in K_{ATP} channel density distribution in the cocultured cells, possibly influenced by signals arising from the tumor cells. An increased [^{3}H]-glibenclamide binding in GBM compared with normal brain tissue was also observed (Fig. 6*A*). RT-PCR analysis also showed the influence of tumor cells on K_{ATP} channel mRNA expression in endothelial cells cocultured with RG2 and GBM primary cells (Fig. 6*B*). Western blot analysis (Fig. 6*C*) and K_{ATP} channel activity assay by potentiometry (Fig. 6, *D* and *E*) confirmed the RT-PCR results and also indicated that tumor cells may induce overexpression of K_{ATP} channels in endothelial cells.

K_{ATP} Channel Activity. The functional activity of putative K_{ATP} channels in a monolayer of HBMVECs and GBM cells was determined by measuring their membrane potential in response to MS at various concentrations. The depolarization action of MS was highly pronounced in GBM when compared with HBMVECs (Fig. 6*D*). The membrane potential decreases in HBMVECs and GBM cells in response to the addition of MS, and a return to resting membrane potential with the addition of glibenclamide was measured spectrofluorometrically using potassium ion-fluorescent dye. These results demonstrate K_{ATP} channel activity in HBMVECs and GBM cells in response to MS. Furthermore, endothelial cells, when cocultured with tumor cells, exhibited higher activity than endothelial or tumor cells alone (Fig. 6*E*). This finding suggests the presence of higher K_{ATP} channel density distribution on endothelial cells that were grown with brain tumor cells than on endothelial cells alone. This observation confirmed similar results obtained by RT-PCR, Western blot, and [^{3}H]-glibenclamide binding experiments (Fig. 6*A*). In other experiments, we observed that MS caused a dose-dependent decrease in K^+ dye-specific fluorescence intensity in RBECs, HBMVECs, and RG2

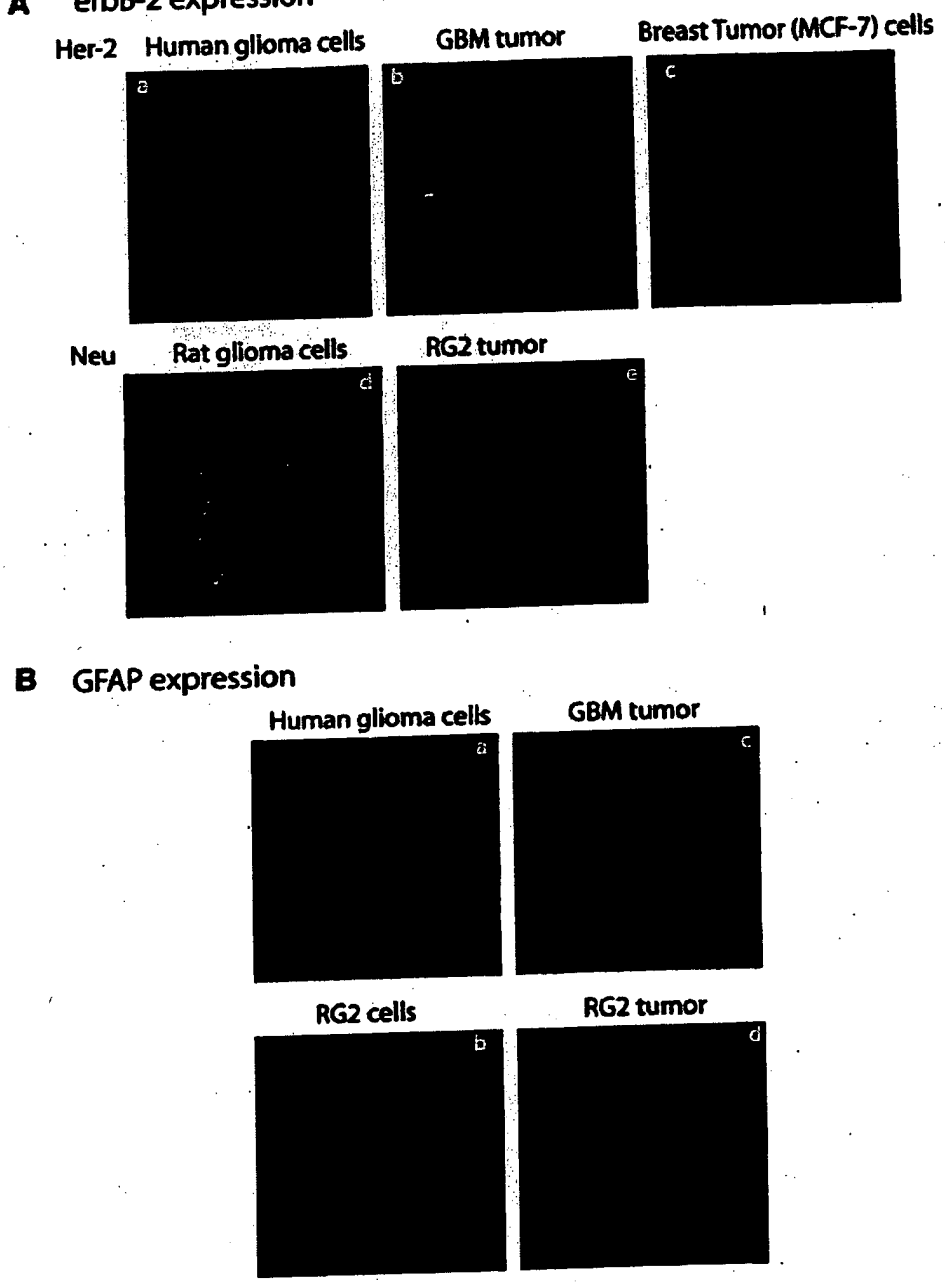

Fig. 4. ErbB-2 and glial fibrillary acidic protein (*GFAP*) expression. *A*, overexpression of erbB-2 receptors was demonstrated using Her-2 and Neu antibodies *in vitro* in human (*a*) and rat (*d*) cells and *in vivo* in GBM (*b*) and RG2 (*e*) tumor, respectively. Low erbB-2-expressing MCF-7 cells were used as a negative control (*c*). *B*, the glial origin of GBM and RG2 was demonstrated with GFAP immunostaining *in vitro* and *in vivo*.

and GBM cells that was reversed by glibenclamide administration (data not shown).

Immunolocalization of K_{ATP} Channels. We next asked why and where K_{ATP} channel modulators selectively induce BTB permeability without affecting BBB permeability. Our hypothesis was that such a selective effect might be because of increased expression of K_{ATP} channels in tumor capillaries and tumor cells compared with normal brain. To address this issue, we used anti-$K_{ir}6.2$ subunit (the pore-forming) antibody to immunolocalize K_{ATP} channels in paraformaldehyde perfusion-fixed GBM and RG2 tumor-bearing rat brain sections. This analysis of rat brain or human brain tumor sections for expression of K_{ATP} channels and endothelial cell marker vWF by two-color immunocytochemistry indicated that vWF-positive tumor vessels (red) were also positive for K_{ATP} channels (green). We demonstrated K_{ATP} channel expression on the plasma membrane of endothelial, RG2, and GBM cells. We also observed more intense immunostaining for K_{ATP} channels in tumor cells (Fig. 7*Ab*) compared with normal endothelial cells (Fig. 7*Aa*). Microvessels positive for both antigens are shown in yellow (Fig. 1 and Fig. 7, *B* and *C*). We also sought to determine whether K_{ATP} channels are expressed differentially and more abundantly on tumor cells than in normal brain, which might explain the MS-induced-selective BTB permeability increase. The immunolocalization study of normal brain sections showed some positive expression for K_{ATP} channels in noncapillary cells, whereas no positive K_{ATP} channel expression was observed in normal human brain endothelial cells (Fig. 1). However, a robust expression of K_{ATP} channels in GBM cells (Fig. 7*Ab*), tumor capillary endothelium (Fig. 7, *Bb* and *Bc*), and rat brain tumor capillary endothelial cells (Fig. 7, *Cb* and *Cc*) compared with low K_{ATP} channel colocalization in normal brain capillaries (Fig. 1) was observed. These

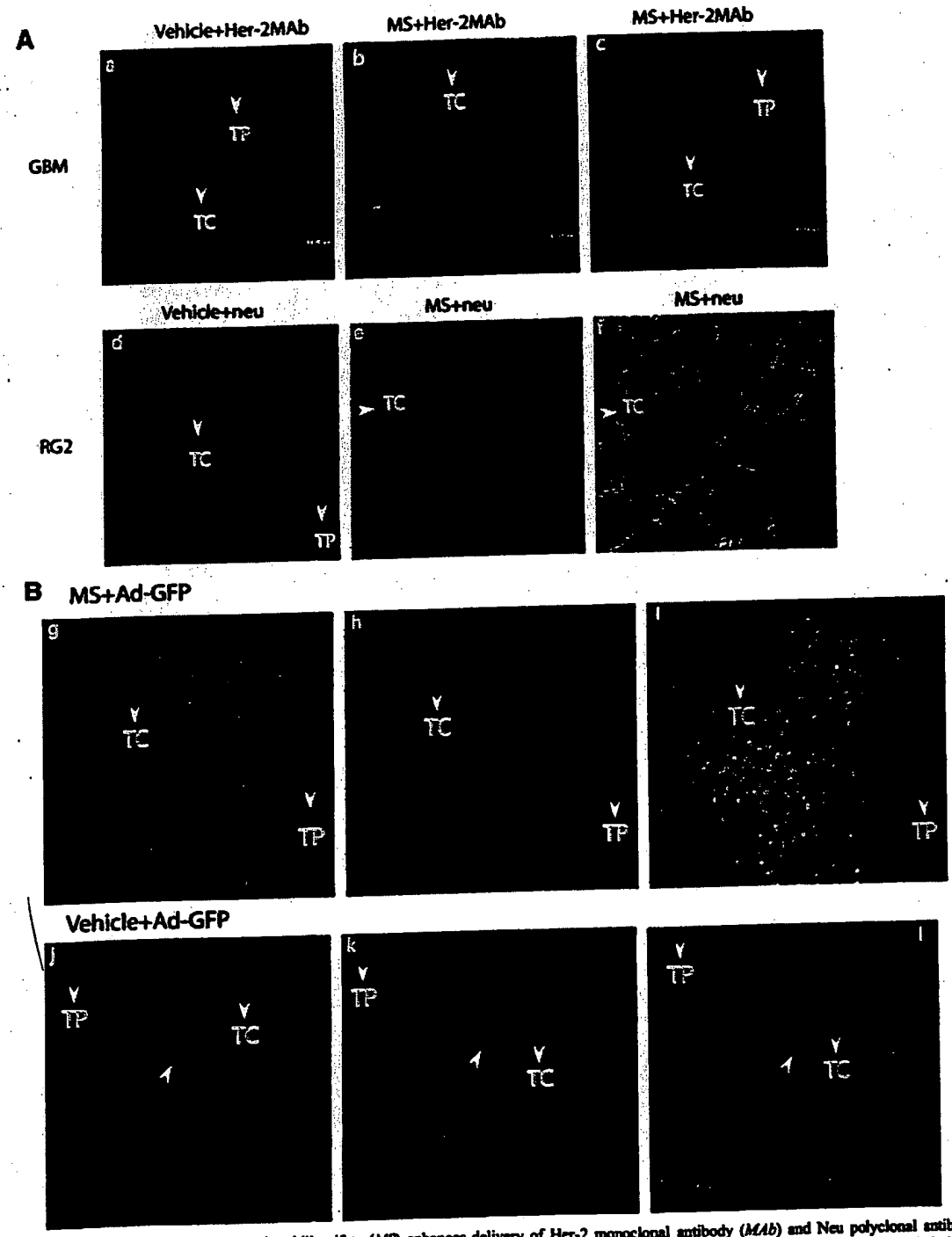

Fig. 5. Increased delivery of macromolecules. *A*, minoxidil sulfate (*MS*) enhances delivery of Her-2 monoclonal antibody (*MAb*) and Neu polyclonal antibody to intracranial glioblastoma multiforme (*GBM*) xenograft and rat glioma (*RG2*) tumor models when infused (intracarotidally) in combination compared with erbB-2 antibody infusion alone. Abundant Her-2 MAb binding was observed in the tumor center (*TC*) and tumor periphery (*TP*), suggesting that infiltrating tumor cells overexpress Her 2 receptors. Enhanced delivery of Neu was observed in TC when coinfused with MS (*e* and *f*), whereas a small amount of Neu was delivered when infused alone. *B*, the ability of MS to increase delivery of adenoviral-green fluorescent protein (*Adv-GFP*) across the blood-tumor barrier was studied in nude rats with intracranial GBM xenografts. Abundant GFP expression was seen predominately in the TC and to a small extent in the TP (*g*). GBM cells are shown expressing glial fibrillary acidic protein (*GFAP*; *h*), and GFP was colocalized with GFAP, suggesting that GFP was predominantly expressed on tumor cells (*i*). In vehicle- and Adv-GFP-infused rat, however, hardly any GFP expression was detected on tumor cells but was observed trapped in blood vessels (*j*), possibly because of the inability of Adv-GFP to cross the blood-tumor barrier. Furthermore, GFP did not colocalize with GFAP on tumor cells, indicating that Adv-GFP did not infect tumor cells.

results strongly suggest that the selective BTB permeability effects of MS and MS-induced BTB permeability increase attenuation by glibenclamide is attributable to the increased density distribution of K_{ATP} channels on brain tumor capillary endothelium and tumor cells compared with normal brain capillary endothelium and normal brain cells.

Vesicular Transport. We further investigated whether vesicular transport is largely responsible for enhanced delivery of drugs and macromolecules across the BTB. We used transmission electron microscopy to demonstrate that no changes occurred in the normal capillary endothelium of contralateral brain tissue after i.c. MS infu-

Table 1 *Physiological measurements during blood-brain tumor barrier (BTB) permeability determination*

The physiological parameters were determined either before or during vasomodulator infusion. The values are mean ± SD of $n \geq 4$ rats, except for cerebral blood flowmetry (CBF), in which three representative rats per group were used. A dose of MS and NS-1619 (30 μg/kg/min) that did not appreciably alter mean arterial blood pressure (MAP) in rats was selected for BTB permeability studies by quantitative radiographic method.

Groups	pH	PaO_2 (mm Hg)	$PaCO_2$ (mm Hg)	MAP (mm Hg)	CBF (% change *vs.* vehicle)
Vehicle	7.3 ± 0.02	87 ± 4	42 ± 2	90 ± 5	0
MS-treated (30 μg/kg/min)	7.38 ± 0.01	90 ± 5	45 ± 1	83 ± 4	8 ± 3
MS + Glb (30 + 10 μg/kg/min)	7.35 ± 0.02	88 ± 5	49 ± 3	81 ± 5	5 ± 2
MS + NS-1619 (both 30 μg/kg/min)	7.30 ± 0.02	92 ± 5	44 ± 4	75 ± 5	12 ± 2

[a] pH, arterial pH; PaO_2, arterial oxygen; $PaCO_2$, arterial carbon dioxide; MS, minoxidil sulfate; Glb, glibenclamide.

sion (data not shown). Similarly, PBS infusion in tumor-bearing rats did not elicit changes in tumor capillary endothelium (Fig. 8*A2*), although i.c. MS infusion accelerated formation of pinocytotic vesicles by invaginations of the luminal membrane of tumor capillary endothelium (Fig. 8*A4*) and the alignment and movement of vesicle arrays along the luminal-abluminal axis of the capillary endothelium. These vesicles dock and fuse with basement membrane and then appear to release their contents on the abluminal side of the endothelial membrane (Fig. 8*A4*). MS significantly increased vesicular density (Fig. 8*B*), although MS did not alter the endothelial tight junction indices in the tumor capillaries (Fig. 8*C*). These results demonstrate for the first time that the primary cellular mechanism for macromolecular delivery across the BTB after K_{ATP} channel activation is via increased vesicular transport and not via the paracellular route through endothelial tight junctions.

Survival Study. In Wistar rats harboring intracranial RG2 tumors, we demonstrated that MS significantly enhanced [^{14}C]-CPN delivery to tumor without affecting normal brain (Fig. 3*B*). In addition, Kaplan-Meier analysis showed that rats with RG2 tumors survived significantly longer when treated with CPN and MS in combination compared with CPN only and vehicle only groups (Fig. 9*A*). The mean survival in the MS and CPN-treated group was 90.57 ± 6.56 days ($P < 0.01$ *versus* CPN alone; $P < 0.001$ *versus* untreated group) compared with the CPN alone group (55.46 ± 5.71 days) and the untreated group (29.56 ± 2.44 days). Combination treatment (MS + CPN) resulted in a significant reduction in tumor size (Fig. 9*B*). The mean tumor size in the combined treatment (1.30 ± 1.2 mm^2) group was the smallest of any group, followed by the group treated with CPN only (5.30 ± 1.2 mm^2) and the vehicle-treated group (9.37 ± 2.2 mm^2; Fig. 9*A*).

DISCUSSION

Therapy for Brain Tumors. The prognosis for patients with GBM is extremely poor, with a median survival of 9 months, primarily because of a paucity of effective treatment options. Although some agents have proved effective against tumors outside the brain, impaired drug delivery across the BTB limits drug delivery to primary (27) and metastatic brain tumors (28). Usually, primary brain tumors are intrinsically resistant to drugs, only adding to the problem of inadequate drug delivery across the BTB. In contrast, metastatic brain tumors that spread to the brain are sensitive to anticancer drugs. The BTB, however, prevents the delivery of anticancer drugs in sufficient amounts to achieve any therapeutic benefit. Therefore, improved delivery of anticancer agents to brain tumors may greatly improve the prognosis of patients with metastatic brain tumors. Breast and lung cancers, which together account for 70% of brain tumor metastases, are the most frequent tumors to spread to the brain. Some 70% of patients with non-small cell lung cancers respond to chemotherapy using CPN/etoposide. The response rate, however, drops to 10–30% for non-small cell lung cancer patients with brain metastases, because the anticancer drugs fail to reach the tumor in the brain. Delivering

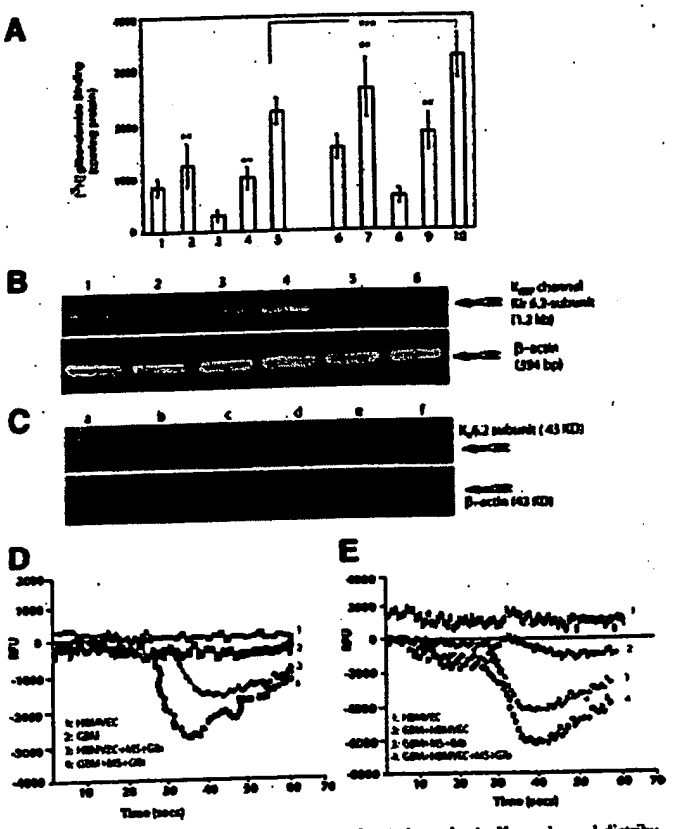

Fig. 6. ATP-sensitive potassium channels (K_{ATP}) channels. A, K_{ATP} channel distribution in membranes prepared from rat brain tissue (*1*), rat glioma tissue (*2*), rat brain endothelial cells (*3*), rat glioma cells (*4*), coculture of rat glioma and rat brain endothelial cells (*5*), human brain tissue (*6*), glioblastoma multiforme tissue (*7*), human brain microvascular endothelial cells (*8*), glioblastoma multiforme cells (*9*), and coculture of human brain microvascular endothelial cells and glioblastoma multiforme cells (*10*). The membranes were incubated with [^{3}H] glibenclamide. The [^{3}H] glibenclamide binding (cpm/mg protein) is significantly greater in cocultures (***, $P < 0.001$) compared with normal and tumor cells. Tumor cells and tumor tissue also exhibit significantly (**, $P < 0.01$) greater [^{3}H] glibenclamide binding compared with endothelial cells and normal brain tissues, respectively. *B*, reverse transcription-PCR analysis of K_v6.2 subunit. *Lane 1*, human brain microvascular endothelial cells; *Lane 2*, primary glioblastoma multiforme cells; *Lane 3*, glioblastoma multiforme cells cocultured with human brain microvascular endothelial cells; *Lane 4*, rat glioma cells cocultured with rat brain endothelial cells isolated from neonatal rat brain; *Lane 5*, rat brain endothelial cells; *Lane 6*, rat glioma cells. Also shown are the intensities of a β-actin band in the same reverse transcription-PCR to ascertain mRNA-loading variance. *C*, immunoblot analysis of SDS-PAGE-fractionated samples (20 μg protein/lane) reveal differential expression of K_{ATP} channel protein immunoreactive with an antipeptide antibody specific for K_v6.2 subunit. *Lane a*, glioblastoma multiforme cells; *Lane b*, human brain microvascular endothelial cells; *Lane c*, glioblastoma multiforme cells cocultured with human brain microvascular endothelial cells; *Lane d*, rat brain endothelial cells; *Lane e*, rat glioma cells; *Lane f*, rat glioma cells cocultured with rat brain endothelial cells. Also shown are the intensities of a M_r 43,000 β-actin band in the same immunoblot to ascertain protein-loading variance. *D*, changes in relative fluorescence intensity (*RFU*) during a 60-s period in response to the addition of minoxidil sulfate (*MS*) and glibenclamide (*Glb*) at 20 and 40 s, respectively, in human brain microvascular endothelial cells (*HBMVEC*; *3*) and glioblastoma multiforme (*GBM*; *4*) cells. In contrast, no change in RFU was observed in HBMVECs and GBM cells without the addition of 1 μM MS and 2 μ M Glb (*1* and *2*). *E*, K_{ATP} channel activity in HBMVEVCs cocultured with GBM cells (*4*) in response to MS was greater compared with the activity of GBM alone (*3*). Control experiments were performed with HBMVECs alone (*1*) and GBM+HBMVEC (*2*) without the addition of MS and Glb. The decrease in fluorescence intensity corresponding to membrane potential changes is plotted on the Y axis as RFU. The addition of Glb reversed the membrane potential to resting values. Note that the MS-induced K_{ATP} channel activity is greater in cocultures than in endothelial or tumor cells alone.

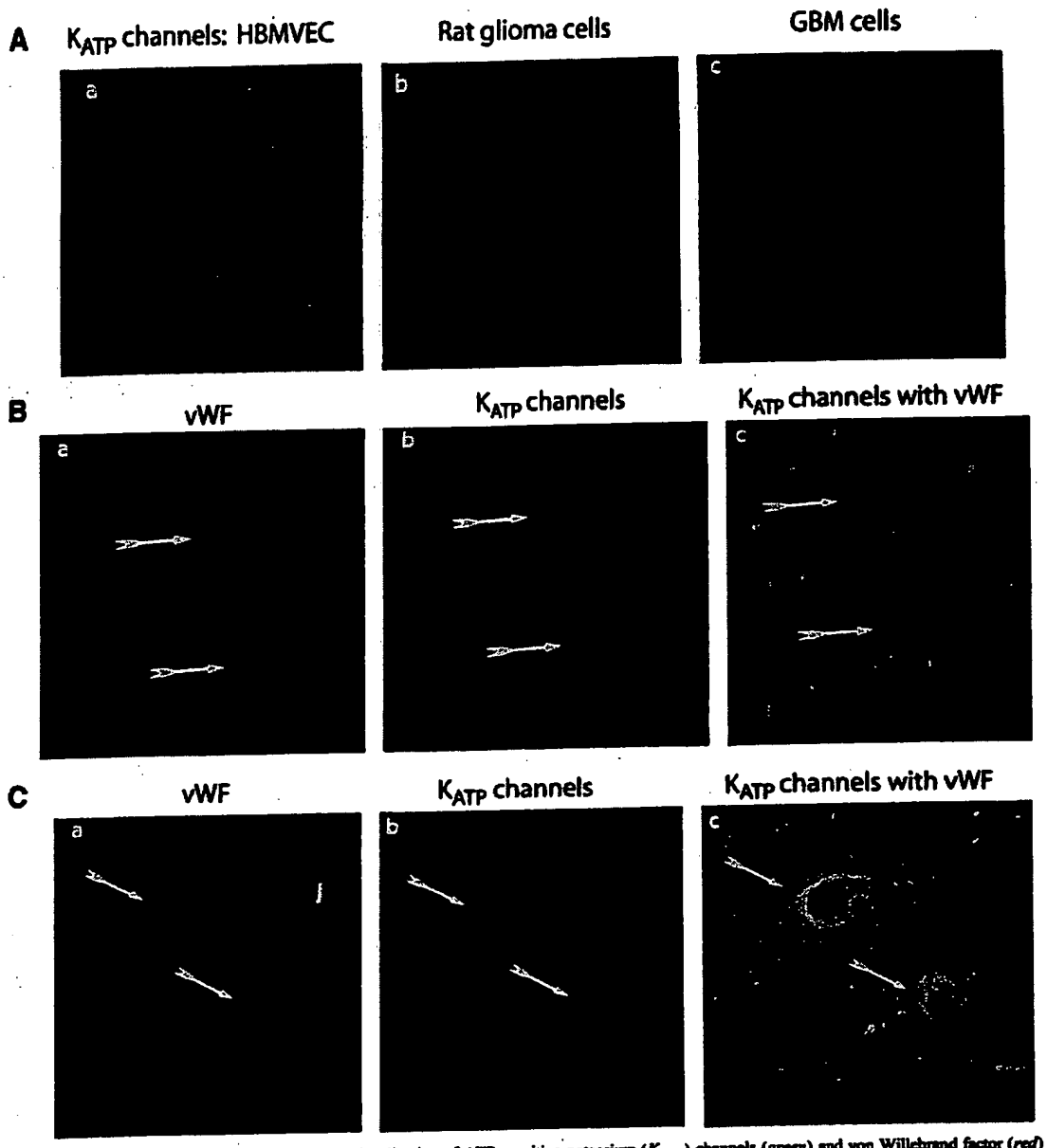

Fig. 7. Immunocytochemistry. A, confocal microscopic immunolocalization of ATP-sensitive potassium (K_{ATP}) channels (*green*) and von Willebrand factor (*red*) in human brain microvascular endothelial cells (HBMVECs; *a*), glioblastoma multiforme (*GBM*) cells (*b*), and rat glioma cells (*c*). *B*, colocalization of K_{ATP} channels in GBM tissue section. Representative image (×20) of a GBM tissue section showing the number of capillaries immunostained with von Willebrand factor (*vWF*; *Ba*), the abundant expression of K_{ATP} channels (*green*) in tumor microvessel as well as in tumor cells (*Bb*), and these K_{ATP} channels colocalizing (*Bc*) with vWF on capillary endothelial cells (*yellow*). C, Similarly, colocalization of K_{ATP} channels in rat glioma tumor capillary endothelial cells was also observed. Control experiments were performed with secondary antibody but without primary antibody.

anticancer drugs to the brain is far more difficult than delivering such drugs to elsewhere in the body. For example, 88% of patients with Her-2-positive breast cancer develop bone metastasis, and 33% develop brain metastasis. When breast cancer patients receive Trastuzumab, however, only 4% develop bone metastasis but 28% still develop brain metastasis. The difference, for the most part, is because of the difficulty of delivering anti-cancer drugs across the BBB/BTB to brain tumor.

BBB Capillaries Differ from BTB Capillaries. We demonstrated that brain tumor capillaries overexpress K_{Ca} channels (4, 5). These findings are consistent with other studies that showed overexpression of vascular proteins (29, 30), such as angiogenic vascular endothelial growth factors, fibronectin, and $\alpha v\beta$ integrins in tumor capillaries. The BTB is structurally and functionally different from the BBB (3, 10, 31, 32). Recent studies (4, 5, 32), however, have shown that certain proteins are specifically expressed or overexpressed in tumor capillaries. We showed that tumor cells overexpress BK type 2 receptors (14), whereas both tumor and tumor capillary endothelial cells overexpress K_{Ca} channels (4, 5) and protein kinase-G (32) and K_{ATP} channels, rendering them potential targets for biochemical modulation of BTB permeability. Although, the magnitude of differences in protein expression between normal and tumor capillaries is qualitative, using a series of complimentary studies, we quantitatively showed that tumor capillaries have increased K_{ATP} channel density, activity, and/or responsiveness to MS.

Biochemical Modulation of BTB Permeability. Vasomodulators, such as BK, nitric oxide donors, soluble guanylate cyclase activators, and NS-1619, increase BTB permeability via K_{Ca} channels (4). MS

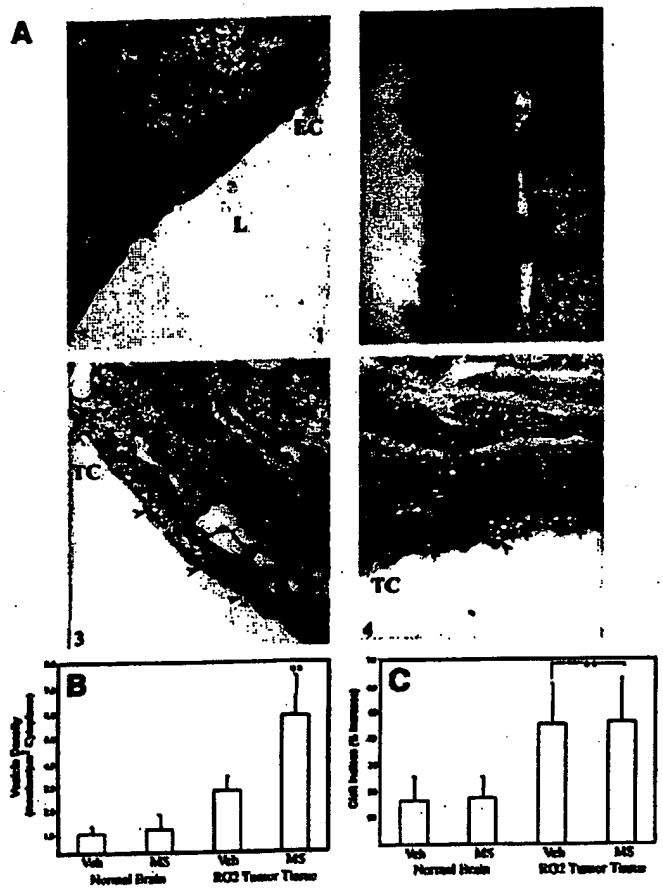

Fig. 8. Mechanism of increased transport. A, induction of vesicular transport in rat glioma tumor capillary endothelium and tumor cells *in vivo*. *1*, in the vehicle-infused rat group, brain tumor microvessel endothelial cells (*EC*) show few vesicles (*arrowheads*) *2*, minoxidil sulfate infusion caused an increased formation of vesicles (*arrows*) by luminal membrane (*L*) invaginations. These vesicles, with an average diameter of 80–90 nm, dock and fuse with the basal membrane (*BM*) *3*, few vesicles are seen in vehicle-infused rat tumor cells (*TC*). *4*, minoxidil sulfate, however, significantly increased the number of pinocytotic vesicles (*arrowheads*) in TC. Values are mean ± SD (n = 5 capillaries/rat). *B*, minoxidil sulfate (MS) induced accelerated formation of transendothelial pinocytotic vesicles in tumor capillary endothelium without affecting endothelial tight junctions. *C*, the number of vesicles in rat glioma (*RG2*) tumor was significantly different from the vehicle (*Veh*)-treated group (••, $P < 0.01$) in tumor capillaries. Cleft indices (percentage) in RG2 tumor capillaries are significantly (••, $P < 0.01$) different from either Veh- or MS-treated normal brain capillaries.

can also biochemically modulate K_{ATP} channels, resulting in a significant BTB permeability increase, allowing the delivery of hydrophilic tracers, GFP-Adv vectors, and Her-2 antibodies specifically to brain tumor tissue. Glibenclamide coinfusion with MS attenuated MS-induced BTB permeability. The efflux of glibenclamide may be a potential concern because it is a substrate for multidrug resistance-associated proteins present on brain tumor microvessels. In our study design, however, glibenclamide efflux should not significantly affect the blockage of MS-induced BTB permeability increase, because glibenclamide was coinfused with MS for only a 15-min period. Furthermore, there was a synergistic effect between K_{Ca} and K_{ATP} channel agonists, suggesting that they act independently. The absence of any significant functional alteration in CBF by K_{Ca} (4) and K_{ATP} (33) channel agonists is consistent with our present data (Table 1). The low doses of these vasoactive agents required to achieve a significant change in permeability allows increased drug delivery without the hypotensive effect expected with high doses of these agents. MS, when administered i.v., also increased BTB permeability in a rat brain tumor model (data not shown) without increasing BBB permeability. On the basis of these observations, we are developing an i.v. formulation of MS to test whether MS enhances anticancer drug delivery to brain tumors in human patients.

K_{ATP} Channels in Cerebral Vasculature. The presence and role of K_{ATP} channels in normal cerebrovascular endothelium have been described (34, 35), but their role in BBB/BTB permeability has not been elucidated. We demonstrated (Figs. 6, *A* and *B*, and 7) abundant expression of K_{ATP} channels in tumor cells (*in vitro* and *in vivo*) in contrast to normal astrocytes and endothelial cells. Confocal images clearly revealed K_{ATP} channel overexpression on tumor and endothelial cells compared with normal brain. Importantly, the tumor capillaries (Fig. 7, *B* and *C*) showed abundant expression of K_{ATP} channels as the K_{ATP} channels colocalize with vWF in tumor capillary endothelial cells. Increased presence of K_{ATP} channels (Fig. 6, *A–C*) and their activity in endothelial cells (Fig. 6, *D* and *E*), possibly by tumor cell-induced signaling, was observed when endothelial cells were cocultured with tumor cells. Others also reported increased K_{ATP} channel activity in pathological conditions such as hypoxia (36), which might also be true in tumors, because tumors thrive in hypoxic environments. In normal brain capillaries, however, K_{ATP} channels were barely detectable even when overexpressed K_{ATP} channels were detected in tumor capillaries (Fig. 1). This unique feature of tumor capillaries offers a mechanism that can be exploited to alter tumor capillary permeability selectively without concomitant effects on normal brain.

Brain Tumor Cells Induce K_{ATP} Channel Overexpression. In rat and human brain tumor cells, we demonstrated the functional activity of K_{ATP} channels in normal and tumor cells alone and in cocultures. MS elicited higher membrane potential changes in tumor cells than in normal cells, suggesting greater density distribution or sensitivity of K_{ATP} channels in tumor cells than in normal cells. Furthermore, *in vitro* [^{3}H]glibenclamide binding studies showed that K_{ATP} channel density is significantly higher in tumor than normal cells/tissue (Fig. 6*A*). We concluded that K_{ATP} channels are overexpressed both on tumor cells and tumor capillary endothelial cells compared with those of normal brain, which might explain why MS selectively increases BTB permeability while leaving the BBB unaffected. Because of the observed synergistic effect of K_{Ca} and K_{ATP} channel agonists on BTB permeability, K_{ATP} channels are an additional target besides K_{Ca} channels (4, 5) for BTB permeability modulation to enhance drug delivery to brain tumors.

Mechanism of Increased Transport: Pinocytotic Vesicles or via Tight Junctions? Our results demonstrate that MS induces accelerated formation of transport vesicles in both brain tumor capillary endothelium and tumor cells by MS-induced activation of K_{ATP} channels (Fig. 8*A*). Therefore, vesicular transport is largely responsible for enhanced delivery of drugs across the BTB rather than via the opening of endothelial tight junctions. This finding is consistent with our previous study (4), in which we reported that a K_{Ca} channel agonist, NS-1619, increased the density of rat brain tumor microvessel endothelial vesicles. A slight increase in basal BTB permeability might be attributable to a small increase in the number of pinocytotic vesicles and the tight junctional cleft index (37, 38). We found a direct relationship between an increase in the number of brain tumor capillary endothelial vesicles and increased BTB permeability. Importantly, we observed that rat brain tumor capillary endothelial cells form far more vesicles (Fig. 8*B*) than normal brain capillary endothelial cells without altering the endothelial tight junctions in response to vasomodulators, such as MS (Fig. 8*C*) or NS-1619 (4).

Enhanced Survival. In a previous study, we showed that CPN enhanced survival when rats with intracranial glioma were cotreated with NS-169 (5) or BK (13). In the present study, we showed that i.c. MS infusion selectively enhanced [^{14}C]carboplatin delivery to tumor tissue without increasing delivery to normal brain (Fig. 3*B*). We also

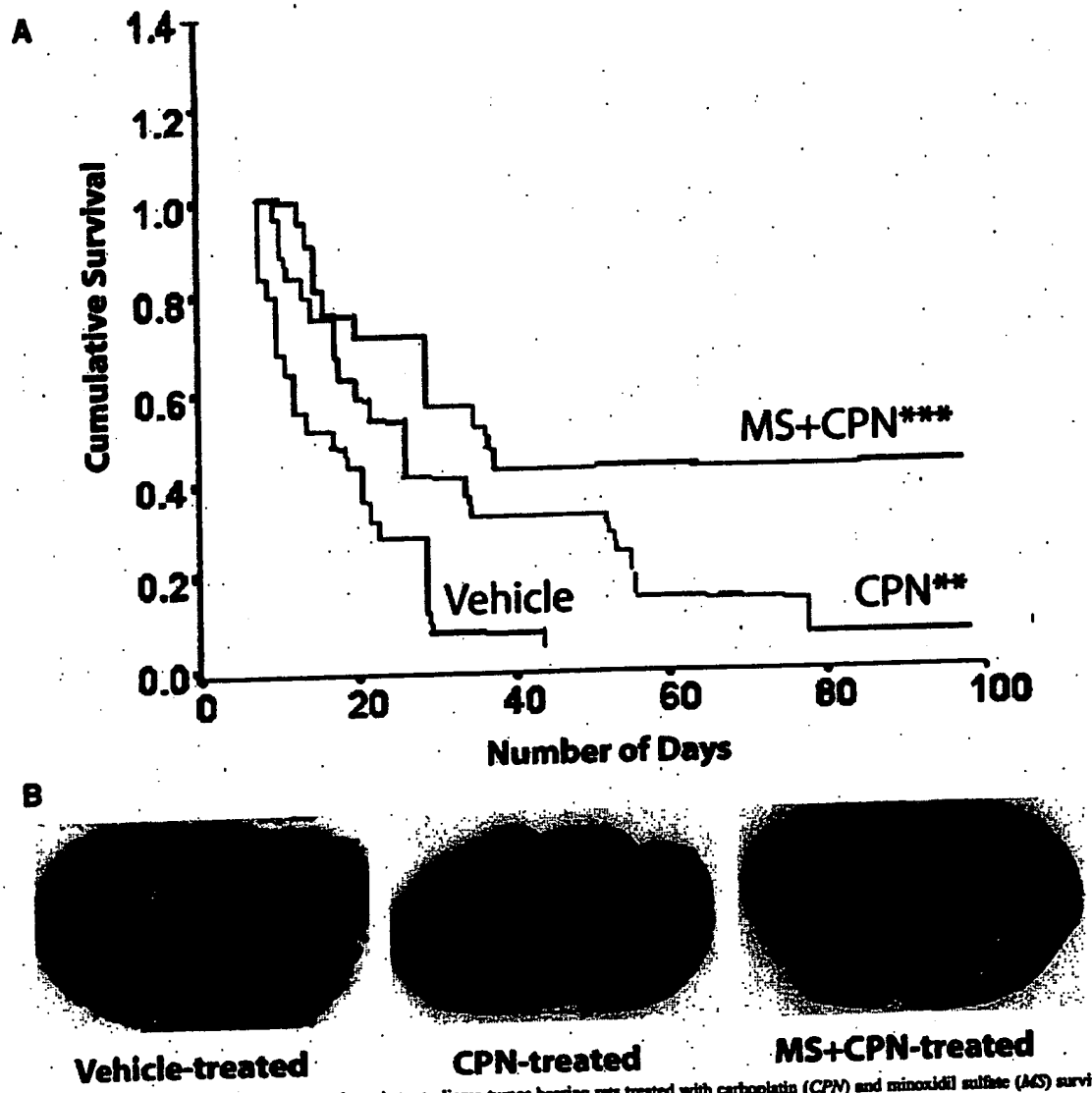

Fig. 9. Survival study. A, Kaplan-Meier survival curves show that rat glioma tumor-bearing rats treated with carboplatin (*CPN*) and minoxidil sulfate (*MS*) survived significantly (**, $P < 0.001$; $n = 30$) longer than vehicle-treated rats. Rats treated with CPN alone also survived significantly (**, $P < 0.01$; $n = 26$) longer than the untreated group. In a randomized study, three groups of rats were treated i.c. (once a day for 3 consecutive days) via an exteriorized catheter 7 days after tumor implantation. *B*, representative coronal sections obtained from rats from each group were stained with H&E. In an untreated group, the brain sections of rats that died on day 25 had huge tumors (9.37 ± 2.2 mm^2) compared with rats that received either CPN (5.30 ± 1.9 mm^2) alone or MS with CPN (1.30 ± 1.2 mm^2). Notice a necrotic tumor area in a representative rat brain section after MS and CPN infusions. This suggests increased CPN delivery and, therefore, enhanced cytotoxicity of the drug.

showed that MS coinfusion with CPN in rats resulted in tumor regression, significantly increasing survival (Fig. 8, *A* and *B*). Primary brain tumors, particularly GBM, frequently have altered genes resulting in tumor cell proliferation, as well as poor prognosis and patient survival (39–41). In particular, Her-2, which is overexpressed in 17–20% of GBMs (20), facilitates tumor cell proliferation (41). Trastuzumab and 2C4, developed by Genentech Inc., are potential Her-2 receptor-based therapies for gliomas. The molecular sizes of these MAbs, however, prevent their efficient delivery across the BTB to tumor. We demonstrated that MS-induced biochemical modulation of K_{ATP} channels enhanced delivery of macromolecules, including Her-2 MAb, selectively to tumors without increasing MAb delivery to normal brain in a GBM xenograft model (Fig. 5*A*). This finding suggests that therapeutic antibodies could be efficiently and selectively directed to glioma cells *in vivo*. In addition to MAb therapy, gene therapy for GBM is emerging as a potential treatment strategy. Efficient and selective delivery of adenoviral vectors to tumor across the BTB, however, is difficult when viral products are administered through an intravascular route because of the difficulty of getting such vectors across the BTB. In this study, we demonstrated enhanced and selective GFP-Adv delivery across the BTB after i.c. coinfusion with MS (Fig. 5*B*). This strategy may be useful clinically to deliver therapeutic antibodies with a chemotherapeutic drug or gene product selectively to brain tumor while leaving healthy brain intact.

Summary. Taken together, our findings demonstrate that K_{ATP} channels are effective targets for BTB permeability modulation. It is conceivable that other types of potassium channels may play a role in BTB permeability regulation, which remains to be thoroughly investigated. This study presents evidence that activation of K_{ATP} channels by specific agonists, such as MS, can sustain enhanced drug delivery selectively to tumors. Specifically, we showed that CPN delivery to brain tumors can be increased using MS, resulting in enhanced survival in rats with intracranial tumors. Furthermore, MS-induced BTB permeability modulation allows delivery of macromolecules (such as dextran, Her-2 MAb, and GFP-Adv) selectively to brain tumor. In conclusion, our results confirm that selective and enhanced delivery of

small and macromolecules across brain tumor microvessels after K_{ATP} channel activation can be exploited to increase BTB permeability and enhance drug delivery to brain tumor. This study may have significant implications for improving targeted delivery of antineoplastic agents to brain tumors and neuropharmaceutics to diseased brain regions while leaving normal brain unaffected.

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REFERENCES

1. Warnick, R. E., Prados, M. D., Mack, E. E., Chandler, K. L., Doz, F., Rabbitt, J. E., and Malec, M. K. A phase II study of intravenous carboplatin for the treatment of recurrent gliomas. J. Neuro-oncol., *19:* 69–74, 1994.
2. Montemurro, F., Choa, G., Faggiuolo, R., Sperti, E., Capaldi, A., Donadio, M., Minischetti, M., Salomone, A., Vietti-Ramus, G., Alabiso, O., and Aglietta, M. Safety and activity of docetaxel and trastuzumab in HER2 overexpressing metastatic breast cancer: a pilot phase II study. Am. J. Clin. Oncol., *26:* 95–97, 2003.
3. Ruoslahti, E. Specialization of tumor vasculature. Nat. Rev. Cancer, *2:* 83–90, 2002.
4. Ningaraj, N. S., Rao, M., Hashizume, K., Asotra, K., and Black, K. L. Regulation of blood-brain tumor barrier permeability by calcium-activated potassium channels. J. Pharmacol. Exp. Ther., *301:* 838–851, 2002.
5. Ningaraj, N. S., Rao, M., and Black, K. L. Calcium-dependent potassium channels as a target protein in the modulation of blood-brain tumor barrier. Drug News Perspect., *16:* 1–8, 2003.
6. Inamura, T., and Black, K. L. Bradykinin selectively opens blood-tumor barrier in experimental brain tumors. J. Cereb. Blood Flow Metab., *14:* 862–870, 1994.
7. Nakano, S., Matsukado, K., and Black, K. L. Increased brain tumor microvessel permeability after intracarotid bradykinin infusion is mediated by nitric oxide. Cancer Res., *56:* 4027–4031, 1996.
8. Black, K. L., Hoff, Y. R., McGillicuddy, J. E., and Gebarski, S. S. Increased leukotriene C_4 and vasogenic edema surrounding brain tumors in humans. Ann. Neurol., *19:* 592–595, 1986.
9. Black, K. L., Clougbesy, T., Huang, S. C., Gobin, Y. P., Zhou, Y., Grous, J., Nelson, G., Farahani, K., Hoh, C. K., and Phelps, M. Intracarotid infusion of RMP-7, a bradykinin analog, and transport of gallium-68 ethylenediamine tetraacetic acid into human gliomas. J. Neurosurg., *86:* 603–609, 1997.
10. Hashizume, K., and Black, K. L. Increased endothelial vesicular transport correlates with increased blood-tumor barrier permeability induced by bradykinin and leukotriene C4. J. Neuropathol. Exp. Neurol., *61:* 725–735, 2002.
11. Sugita, M., and Black, K. L. Cyclic GMP-specific phosphodiesterase inhibition and intracarotid bradykinin infusion enhances permeability into brain tumors. Cancer Res., *58:* 914–920, 1998.
12. Inamura, T., Nomura, T., Ikezaki, K., Fukui, M., Pollinger, G., and Black, K. L. Intracarotid histamine infusion increases blood tumor permeability in RG2 glioma. Neurol. Res., *16:* 125–128, 1994.
13. Matsukado, K., Sugita, M., and Black, K. L. Intracarotid low dose bradykinin infusion selectively increases tumor permeability through activation of bradykinin B2 receptors in malignant gliomas. Brain Res., *792:* 10–15, 1998.
14. Liu, Y., Hashizume, K., Samoto, K., Sugita, M., Ningaraj, N., Asotra, K., and Black, K. L. Correlation between bradykinin-induced blood tumor barrier permeability and B2 receptor expression in experimental brain tumors. Neurol. Res., *23:* 379–387, 2001.
15. Brayden, J. E. Functional roles of KATP channels in vascular smooth muscle. Clin. Exp. Pharmacol. Physiol., *29:* 312–316, 2002.
16. Kitazono, T., Faraci, F. M., Taguchi, H., and Heistad, D. D. Role of potassium channels in cerebral blood vessels. Stroke, *26:* 1713–1723, 1995.
17. Morimoto, T., Aoyagi, M., Tamaki, M., Yoshino, Y., Hori, H., Duan, L., Yano, T., Shibata, M., Ohno, K., Hirakawa, K., and Yamaguchi, N. Increased levels of tissue endostatin in human malignant gliomas. Clin. Cancer Res., *8:* 2933–2938, 2002.
18. Cobbs, C. S., Brenman, J. E., Aldape, K. D., Bredt, D. S., and Israel, M. A. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res., *55:* 727–730, 1995.
19. Huncharek, M., and Kupelnick, B. Epidermal growth factor receptor gene amplification as a prognostic marker in glioblastoma multiforme: results of a meta-analysis. Oncol. Res., *12:* 107–112, 2000.
20. Forseen, S. E., Potti, A., Koka, V., Koch, M., Fraiman, G., and Levitt, R. Identification and relationship of HER-2/neu overexpression to short-term mortality in primary malignant brain tumors. Anticancer Res., *22:* 1599–602, 2002.
21. Johannessen, L. E., Haugen, K. E., Ostvold, A. C., Stang, E., and Madshus, I. H. Heterodimerization of the epidermal-growth-factor (EGF) receptor and ErbB2 and the affinity of EGF binding are regulated by different Mechanisms. Biochem. J., *356:* 87–96, 2001.
22. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med., *344:* 783–792, 2001.
23. Ohno, K., Pettigrew, K. O., and Rapaport, S. T. Lower limits of cerebrovascular permeability to nanoelectrolytes in the conscious rat. Am. J. Physiol., *253:* H299–H307, 1978.
24. Lorenz, E., Alekseev, A. E., Krapivinsky, G. B., Carrasco, A. J, Clapham, D. E., and Terzic, A. Evidence for direct physical association between a K+ channel (Kir6.2) and an ATP-binding cassette protein (SUR1) which affects cellular distribution and kinetic behavior of an ATP-sensitive K+ channel. Mol. Cell. Biol., *18:* 1652–1659, 1998.
25. Challinor-Rogers, J. L., Kong, D. C., Iskander, M. N., and McPherson, G. A. Structure-activity relationship of [3H]-glibenclamide binding to membranes from rat cerebral cortex. J. Pharmacol. Exp. Ther., *273:* 778–786, 1995.
26. Smith, G. M., Berry, R. L., Yang, J., and Tanelian, D. Electrophysiological analysis of dorsal root ganglion neurons pre-and post-coexpression of green fluorescent protein and functional 5-HT3 receptor. J. Neurophysiol., *77:* 3115–3121, 1997.
27. Fellner, S., Bauer, B., Miller, D. S., Schaffrik, M., Fankhanel, M., Spruss, T., Bernhardt, G., Graeff, C., Farber, L., Gschaidmeier, H., Buschauer, A., and Fricker, G. Transport of paclitaxel (Taxol) across the blood-brain barrier in vitro and in vivo. J. Clin. Invest., *110:* 1309–1318, 2002.
28. Fuchs, I. B., Loebbecke, M., Buhler, H., Stohlenburg-Didinger, G., Heine, B., Lichtenegger, W., and Schaller, G. HER2 in brain metastases: issues of concordance, survival, and treatment. J. Clin. Oncol., *20:* 4130–4133, 2002.
29. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., *1:* 27–31, 1995.
30. Pardridge, W. M. Blood-brain barrier genomics. *In:* W. M. Pardridge (ed.), Brain Drug Targeting: The Future of Brain Drug Development, pp. 275–300. Cambridge, UK: Cambridge University Press, 2001.
31. Asotra, K., and Black, K. L. Blood-brain barrier as portal for drug delivery. Adv. Clin. Neurosci., *10:* 323–339, 2000.
32. Uchida, M., Ningaraj, N., Chen, Z., Black, L., and Asotra, K. Cyclic GMP-induced blood-brain-tumor barrier permeability is not mediated by cyclic GMP-dependent protein kinase. Am. Assoc. Neurol. Surg., 1160, 2001.
33. Esaki, T., Itoh, Y., Shimoji, K., Cook, M., Jehle, J., and Sokoloff, L. Blockade of K(ATP) channels with glibenclamide does not alter functional activation of cerebral blood flow in the unanethetized rat. Brain Res., *948:* 56–63, 2002.
34. Nelson, M. T., and Quayle, J. M. Physiological roles and properties of potassium channels in arterial smooth muscle. Am. J. Physiol., *268:* C799–C822, 1995.
35. Yokoshiki, H., Sunagawa, M., Seki, T., and Sperelakis, N. ATP-sensitive K+ channels in pancreatic, cardiac, and vascular smooth muscle cells. Am. J. Physiol., *274:* C25–C37, 1998.
36. Schoch, H. J., Fischer, S., and Marti, H. H. Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain. Brain, *125:* 2549–2557, 2002.
37. Stewart, P. A. Endothelial vesicles in the blood-brain barrier: are they related to permeability? Cell. Mol. Neurobiol., *20:* 149–163, 2002.
38. Liebner, S., Fischmann, A., Rascher, G., Duffner, F., Grote, E. H., Kalbacher, H., and Wolburg, H. Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. Acta Neuropathol., *100:* 323–331, 2000.
39. Newcomb, E. W., Cohen, H., Lee, S. R., Bhalla, S. K., Bloom, J., Hayes, R. L., and Miller, D. C. Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFr, MDM2 or Bcl-2 genes. Brain Pathol., *8:* 655–667, 1998.
40. Ljubimova, J. Y., Lakhter, A. J., Loksh, A., Yong, W. H., Riedinger, M. S., Miner, J. H., Sorokin, L. M., Ljubimov, A. V., and Black, K. L. Overexpression of α4 chain-containing laminins in human glial tumors identified by gene microarray analysis. Cancer Res., *61:* 5601–5610, 2001.
41. Schlegel, J., Stumm, G., Brandle, K., Merdes, A., Mechtersheimer, G., Hynes, N. E., and, Kiessling, M. J. Amplification and differential expression of members of the erbB-gene family in human glioblastoma. J. Neuro-oncol., *22:* 201–207, 1994.

EXHIBIT B

K_{ATP} channel Localization in Metastatic Brain Tumors

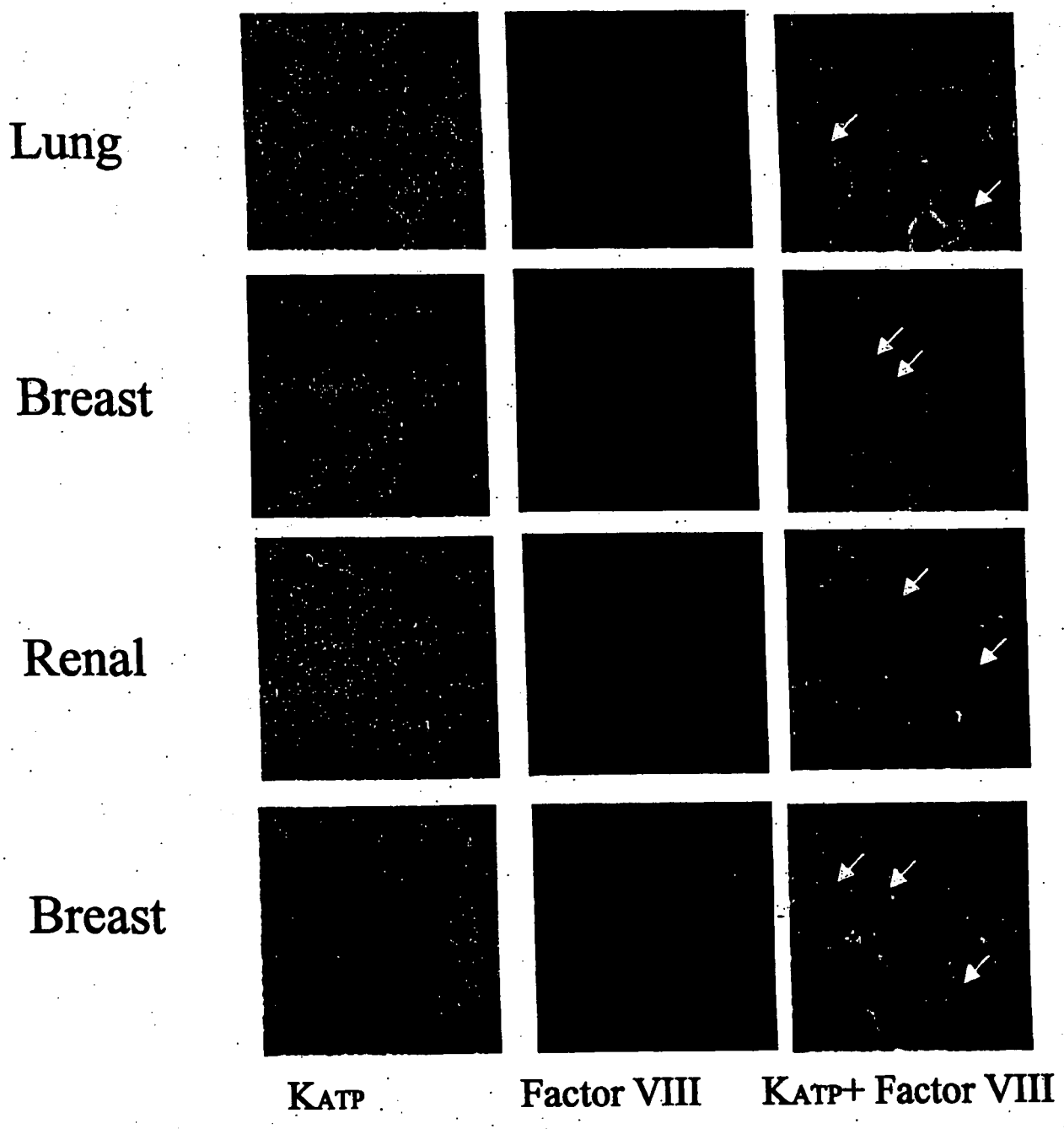

EXHIBIT C

Potassium Channel Agonist (60 μg/kg/min for 15 min, i.v.)-induced BTB permeability Increase in Metastatic Breast (MDA-MB 361) Brain Tumor model

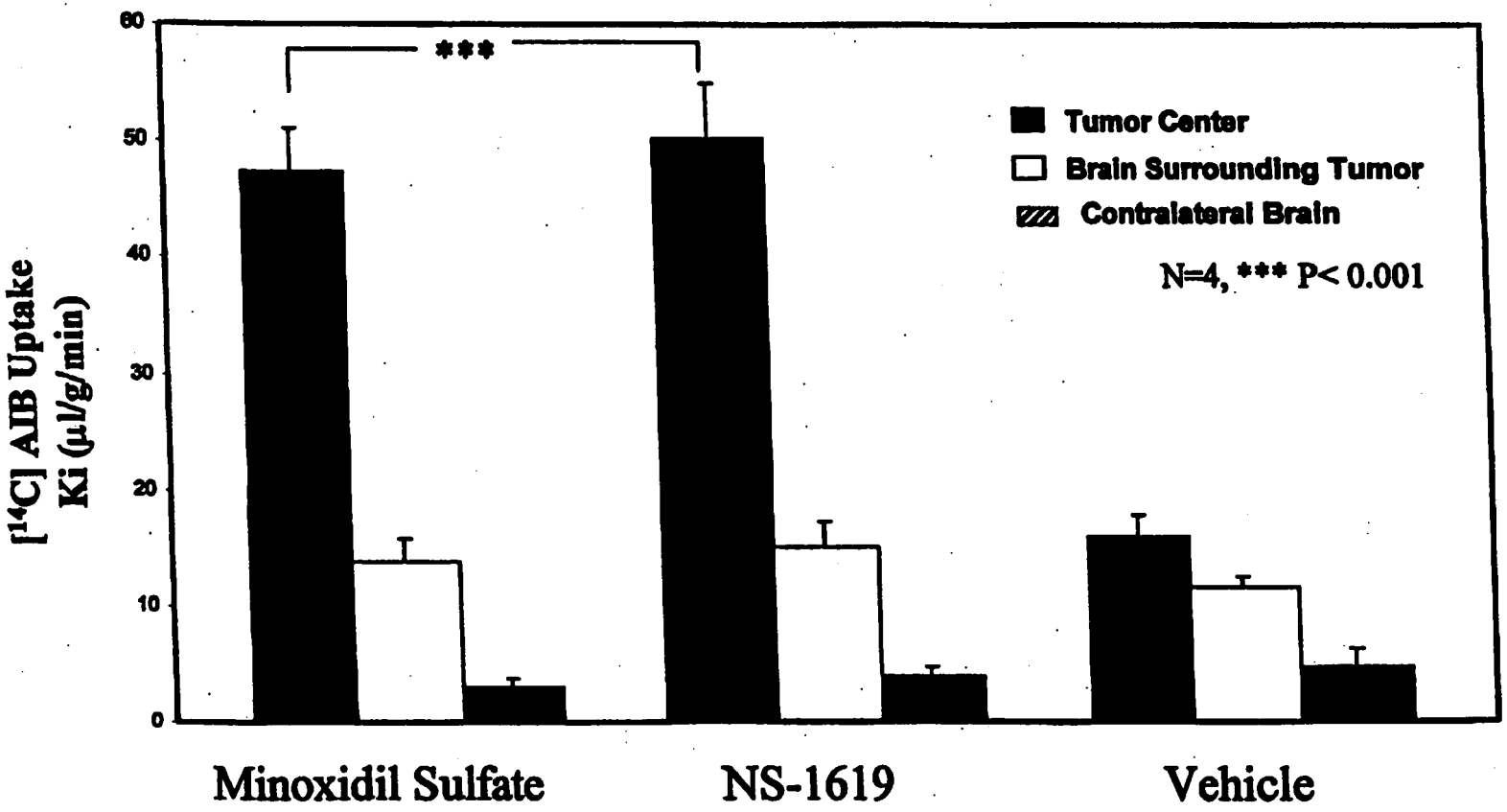

EXHIBIT D

Effect of Minoxidil Sulfate (60 μg/kg/min, i.v. for 15 min) on BTB permeability of [^{14}C AIB] in metastatic NSCLC brain tumor model